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REPRODUCTIVE BIOTECHNOLOGIES: A NEW APPROACH FOR CERVIDS CONSERVATION.

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Introduction

Biodiversity

In the 1992 Earth Summit in Rio de Janeiro world leaders adopted the Convention on Biological Diversity (CBD). It is the most important Convention dealing with biodiversity conservation: it follows the growing threat posed by human activity to biodiversity and it is inspired by the world community's growing commitment to sustainable development.

The Convention has three main objectives:

1.To conserve biological diversity

2.To use biological diversity in a sustainable way

3.To share the benefits of biological diversity fairly and equitably.

The United Nations proclaimed 2010 "International Year of Biodiversity" (Resolution 61/203). Biological diversity - or biodiversity - is the term given to the variety of life on Earth and the natural patterns it forms. Biodiversity includes genetic variation within species, the variety of species in an area, and the

variety of habitat types within a landscape (David J., 2009). General interest in biodiversity has grown rapidly in recent decades, in parallel with the growing concern about nature conservation and as a consequence of accelerating rates of natural habitat loss, habitat fragmentation, degradation, and resulting extinctions of species (Lloyd J., 2009).

The IUCN Red List of Threatened Species (also known as the IUCN Red List or Red Data List), founded in 1963, is the world's most comprehensive inventory of the global conservation status of plant and animal species. It estimates that 12-52% of species within well-studied higher taxa such as vertebrates and vascular plants are threatened with extinction.

Species are classified in nine groups, set through criteria such as rate of decline, population size, area of geographic distribution, and degree of population and distribution fragmentation.

Sardinia is widely considered for its peculiar variety of rare or uncommon animals, such as species of mammals: the Mediterranean Monk Seal (*Monachus monachus*, Hermann, 1779), the Giara horse (*Equus caballus*, Linneo 1758), the Albino Donkey (*Equus africanus asinus* var.albina

Linnaeus, 1758), the Mouflon (*Ovis aries* Linnaeus, 1758, the Sardinian Long-eared Bat (*Plecotus sardus* Mucedda, Kiefer, Pidincedda and Vieth 2002), the Sardinian fox (*Vulpes vulpes ichnusae*, Miller 1907), the wild boar (*Sus scrofa* Linnaeus, 1758), Sardinian wild cat (*Felis lybica sarda*, Lataste 1885) and the Sardinian red deer (*Cervus elaphus corsicanus*, Erxleben 1777).

Nowadays in Sardinia, subspecies *C. e. corsicanus* is strictly protected under Appendix II of the Bern Convention and Annexes II* and IV of the EU Habitats and Species Directive and is included as a Near Threatened on the IUCN Red List (Lovari S., 2011). Population size estimates for Sardinian deer differ among authors, but at present, there may be more than 6000 (Apollonio M, 2010).

Several protection measures were implemented to avoid extinction in recent past but despite the increase in numbers (Ente Foreste della Sardegna 2010; Ciucci et al.2009) there has been no corresponding increase in the spatial distribution of the Sardinian population, which is still limited to the three small areas that were occupied in the 1980s (plus Monte Lerno and Monte Linas where a few individuals have been reintroduced).

Nowadays, the fragmentation of suitable habitat is the major problem in conservation and preservation of genetic variability (Zachos F.E., 2011). To increase conservation options it would be useful the identication of additional sites suitable for reintroductions, for management, and for conservation planning (Puddu G., 2009) and, above all, the establishment of a Genetic Rescue Bank. If used properly, these Banks could preserve the current genetic diversity and give future reproductive opportunities through different techniques (Leon Quinto T., 2008).

ARTs application

Conservation of threatened wildlife species could be carry out through two major approaches. The first is based on the preservation of habitat, generally on a large scale (Margukes and Pressey 2000; Hanks 2001). The second on breeding and propagating individual species ex situ (in captivity; Holt and Pickard 1999; Loskutoff 2003; Pukazhenthi and Wildt 2004). Habitat preservation would be the highest priority but it is not always feasible.

Advanced biotechnologies could be considered an useful instrument to preserve endangered species, in *in situ* and *ex situ* conservation programs. Until now, several techniques have been applied: artificial insemination (AI), non invasive hormone monitoring, in vitro oocyte maturation (IVM), fertilisation (IVF) and culture (IVC), embryo transfer and germplasm banking (Pope 2000; Wildt et al 2001; Monfort et al ,2003; Pukazhenthi and Wildt 2004) and recently cloning and stem cells technologies (Loi P. et al, 2011; Gomez M.C. et al, 2010).

Production of embryo in vitro allows to produce multiple offspring from genetically valuable individuals. Potentially, it could allow more rapid expansion of a population than through natural breeding using also prepubertal and pregnant (Meintjes et al., 1995) females, ovarian tissue excision (Jewgenow K, 2008) by embryo division or from males and females after death(Loskutoff et al., 2003). Moreover, it is possible to control certain pathogens using treatment methods for semen or embryos (Wrathall & Sutmoller, 1998).

In conclusion, the study of reproduction is fundamental for conserving species, populations and, indirectly, the vitality of entire ecosystems. However a

successful management of wildlife depends on intensive genetic and reproductive management.

In our study we deepen into reproductive physiology of cervids in Sardinia and applied several assisted reproductive techniques that could be useful to support conservation strategies for Sardinian red deer and fallow deer. We collected gametes and tissue from males and females after death to produce embryos and set up a genetic resource bank. Moreover, we carried out *in vivo* electroejaculation on free living population to collect semen during breeding and no breeding season.

Cloning

Introduction

In nature, various forms of asexual reproduction exists: budding (jellyfish, corals and tapeworms), fragmentation (worms), and parthenogenesis (some fishes, insects, frogs and lizards). The Parthenogenesis is more rapid than sexual reproduction and permits quick exploitation of available resources. Cloning in biology is the process of producing similar populations of genetically identical individuals that occurs in nature when organisms such as bacteria, insects or plants reproduce asexually. In ART, reproductive cloning, is a technology used to create an exact genetic match of the donor. The first cloning experiments on animals date from the nineteenth century. In 1891 Hans Driesch separated the blastomeres (cells formed in the first stages of embryonic development) of a two-cell embryo of sea urchin mechanically by shaking them in seawater. The cells started to grow independently and formed two whole sea urchins (Driesch, 1891). However, it is only in 1963 that Tong Dizhou transferred the nuclei from cells of a male Asian carp to enucleated eggs of a female Asian carp, and therefore, generated the first cloned fish in the world. In previous decades, researchers had

cloned micro-organisms and nematodes, as well as amphibians. However, before Tong, nobody had ever managed to clone such a complex organism. The first success in mammalian nuclear transfer (mice) used embryonic cells as donors was reported by Illmensee and Hoppe (1981)but nobody could repeat this experiment. Even though some earlier publications had indicated that cultured cells of embryos at an advanced stage of development could be used as donors for nuclear transfer (Sims and First, 1993; Campbell et al., 1996), the birth of Dolly (Wilmut et al., 1997) was required for wide acceptance that it was possible to clone a grown animal by removing the nucleus of a somatic cell from an adult and inserting it into an enucleated egg. Afterwards, cloned mammals have been produced successfully in cattle (Kato Y., 1998), mouse (Wakayama T. , 1998), goat (Baguisi A.,1999), pig (Polejaeva IA, 2000), rabbit (Chesne P., 2002), cat (Shin T., 2002), rat (Zhou Q, 2003), white-tailed deer (Westhusin M., data not published) ,horse (Galli C, 2003), mule (Woods GL, 2003), dog (Lee BC, 2005), ferret (Li Z, 2006), red deer (Berg D.K.), buffalo (Shi D, 2007), capra pyrenaica (Folch J., 2009), camel (Wani N.A., 2010) with different somatic cell types as nuclear donors.

Techniques in brief

The predominant cell type of choice for reprogramming a somatic nucleus is an oocyte arrested at metaphase II. The technique of somatic cell nuclear transfer (SCNT) comprises the removal of the nuclear DNA of a metaphase II stage oocyte (enucleation), the injection of a single cell into the perivitelline space of the enucleated oocyte, followed by electrofusion, chemical activation and *in vitro* culture of embryos. The oocyte cytoplasm can restore totipotency: using nuclear transfer techniques to expose the chromatin (nuclei) to ooplasm, it is possible to reprogramme the DNA and consequently support complete embryonic development.

In standard SCNT the zona pellucida is preserved: is regarded as important in supporting further development until the hatching.

The most common way of inserting somatic cells is by injecting them under the zona pellucid and then using an electric impulse to induce cell membrane fusion between the enucleated oocyte and the somatic cell. Alternative ways include injection of the cytoplasm-free donor nucleus or the whole somatic cell into the

cytoplasm. Earlier attempts to induce fusion with chemical and viral agents have been less successful and would now be rarely used. When the embryo has been reconstructed, it must be activated. It is known that an increase in intracellular Ca2+ concentration is a universal response elicited by the sperm during fertilization of the oocyte (Whitacker and Patel, 1990). Given the importance of the Ca2+ release during fertilization, most of the currently used oocyte activation procedures rely on methods to induce an intracellular Ca2+ increase. The capability of Ca2+ rises to release oocytes from the meiotic arrest is related to the ability to trigger persistent inactivation of maturation promoting factor (MPF) and cytostatic factor (CSF), which is the result of c-mos and mitogen activated protein kinase (MAPK) activity (Lorca et al., 1993; Collas et al., 1993). It was demonstrated that a single Ca2+ rise, which is evoked by most of the currently used activation procedures, causes only a transient decline of MPF and CSF activity, which is not adequate for full oocyte activation (Lorca et al., 1993; Collas et al., 1993). Artificial activation studies have shown that the most effective stimuli are those which promote multiple intracellular calcium peaks (Vitullo and Ozil, 1992). Based on these, alternative methods of activation have been

developed that combine a transient inactivation of MPF obtainable with a single [Ca2+] rise, with a persistent inhibition of MPF, induced by addition of either protein synthesis inhibitors (e.g., CHX; Bos-Mikich et al., 1995) or non-specific kinase inhibitors (e.g., 6-DMAP; Liu et al., 1998a). Protein synthesis inhibitors usually restrict the synthesis or re-accumulation of cyclin B, thereby, blocking the re-synthesis of MPF activity (Presicce and Yang, 1994). Whereas, non-specific protein kinase inhibitors inhibit kinase activity of MPF by inactivating MAPK (Motlik et al., 1998; Gordo et al., 2000).

Activation could be carried out with single agents, including ionomycin, ethanol, calcium ionophore A23187 and strontium; activation with ionomycin and ethanol in combination with either 6-DMAP or CHX + CB. Combined treatments, especially ionomycin + 6-DAMP and ethanol + CHX + CB, have a significant increase in pronuclear formation, cleavage, blastocyst rate and cell number of blastocyst than treatment alone.

Somatic cell

Cloning have been carried out using cumulus, (Kato Y, 1998, 2000;Tani T, 2002), fibroblasts, (Kato Y, 2000; Zakhartchenko V, 1999), ovarian/granulosa, (Gibbons J., 2002, Wells DN 1998, 1999; Edwards 1999; Piedrahita JA, 2000), mammary(Zakhartchenko V, 1999; Kishi M, 2000), muscle, oviduct(Kato Y, 1998, 2000), and uterine (Kato Y, 2000) cell types. Wakayama et al. reported cloned embryos originating from Sertoli or neuronal nuclei. After collection cells could be used immediately or after long term culture(Wakayama T, 1998).

It has been shown that many adult cells are capable of reprogramming and providing for the development to the blastocyst stage: fibroblasts (rabbit—Mitalipov et al., 1998; Dinnyes et al., 1999; Lagutina et al., 1999;cow—Zakhartchenko et al., 1999c; Vignon et al.,1999), mammary gland epithelium (sheep—Wilmut et al., 1997; Zakhartchenko et al., 1998, 1999a, 1999d), granulosa cells (mouse—Wakayama et al., 1998; cow—Collas and Barnes, 1994; Kato et al., 1998; Wells et al., 1999), oviduct epithelium (cow—Kato et al., 1998), and Sertoli and nervous cells (mouse—Wakayama et al., 1998).

Reprogramming

General scheme of nuclear remodeling:

1) nuclear envelope is disintegrated and nuclear chromatin is condensed (Szollosi et al., 1986, 1988; Collas and Robl, 1991a, 1991b; Barnes et al., 1993; Campbell et al., 1993)
2) the chromatin is again decondensed after activation, a new nuclear envelope and pronuclei are formed, and the nucleus is enlarged to the pronucleus volume (Szollosi et al., 1986; Stice and Robl, 1988;Collas and Robl, 1991a, 1991b)

Nuclear reprogramming includes the morphological and molecular changes that nuclei undergo after transplantation into oocyte such as changes in chromatin and gene expression. Is possible not only with oocytes but also with fertilized eggs (or zygotes), showing that the reprogramming factors are still present at this stage(Egli D, 2007). Transfer of somatic nucleus to the oocyte leads to chromatin decondensation and nuclear swelling, histone replacement and DNA

demethylation (similar to the processes observed during the germ line reprogramming) (Kikyo N., 2000). Reprogramming of the nucleus may be complete or partial. In the case of complete reprogramming, one could expect the birth of viable progeny from the reconstructed embryos, while in the case of incomplete or partial reprogramming the development of reconstructed embryos may be arrested at various developmental stages, which may cause loss of embryos in the preimplantation and postimplantation periods and, possibly, early postnatal mortality (Lagutina I, 2001). The degree of remodeling of the transplanted nucleus depends on the cell cycle stage of the recipient oocyte (Collas and Poccia, 1998). When the nucleus is transplanted in the oocyte at a stage between metaphase II and telophase II, it undergoes a complete remodeling (Szollosi et al., 1986, 1988)

Anomalies

Briefly, anomalies may be caused by the following factors

- Inappropriate donor cell or/and recipient oocyte.

- Inappropriate synchrony between the cell cycle phase of donor nucleus and recipient cytoplasm.

- Inadequate reprogramming of the donor genome(The developmental defects in cloned animals are presumed to result, in part, from problems with the fidelity of genomic reprogramming, owing to a failure to erase 'epigenetic memory')(Simonsson S., 2004).

- Inappropriate handling of oocytes, somatic cells and embryos during maturation, various manipulations and cultural techniques causing mechanical osmotic, electrical, toxic, thermal and other types of damage.

Developmental anomalies produced by SCNT after embryo transfer include low pregnancy rates, an unacceptably high level of losses during early and late pregnancy, stillbirths, early postnatal deaths, short life-span, obesity and malformations. So far, these phenomena are poorly understood—a fact that is reflected in the lack of consensus in nomenclature and classification. The frequency of abnormalities in cloned animals that have been generated by

nuclear transfer suggests that nuclear reprogramming is incomplete and that a better understanding of the mechanisms of gene regulation, particularly those of epigenetic memory, is required.

Application

Application possibilities of cloning in research, industry and agriculture are theoretically almost limitless. The greatest potential of farm animal cloning seems to be the biomedical application: Bioreactor, xenotransplantation, disease models but also agriculture application such as copies of animals with highly valued traits, s to produce animals that can reduce negative agricultural effects on the environment. The potential application of adult cloning ranges from multiplying animals to producing a large number of genetically identical animals for research purposes. Dissemination of genetically superior animals would be made easier through the production of several copies of top breeding animals and the distribution of clones to production farms, particularly where access to artificial insemination and other assisted reproductive technologies is limited. Genetic

improvement programs would benefit from cloning embryos derived from juvenile females of high merit, thereby enabling a shorter generation interval.

iSCNT

This technique has been used widely (a) for studies on the mechanisms underlying nuclear-cytoplasmic interactions, (b) in attempts to rescue highly endangered species, and (c) for the production of ntESCs (Tecirlioglu et al., 2006; Beyhan et al., 2007).

Somatic cell nuclear transfer has thus been suggested as a potentially integral part of wildlife conservation programs (Wildt and Wemmer, 1999; Ryder, 2002; Andrabi and Maxwell, 2007), for increasing the population of endangered mammals and to restore the extinct species (Kenneth, 2002).

However, in exotic or endangered species, the lack of oocytes and recipients precludes the use of traditional somatic cell NT, and an approach such as interspecies NT may be the only alternative to produce embryos and offspring. Interspecies somatic cell nuclear transfer (iSCNT) involves the transfer of a

nucleus or cell from one species into the cytoplasm of an enucleated oocyte from another. In 1973 Tong D.Z., for the first time, inserted the DNA of an Asian carp into an egg of a European crucian carp, a related species, and created the first interspecies clone (Tong *et al*. 1973). One of the first attempts of interspecies NT in mammalian, using the enucleated bovine oocyte as recipient cytoplast was reported by Dominko et al. (1999). Following studies using iSCNT have reported development to the blastocyst stage in the transfer of human (Chang KM), sheep, porcine and monkey nuclei into bovine oocytes (Dominko, 1999) and macaque nuclei into rabbit oocytes (Yang CX, 2003). Further iSCNT attempts using the bovine cytoplast and the karyoplasts from other species include somatic cells from pigs (Yoon *et al.*, 2001), saolas (Bui *et al.*, 2002), elands (Damiani), horses (Li *et al.*, 2002;Sansinena *et al.*, 2002; Tecirlioglu *et al.*, 2006), bears (Ty *et al.*, 2003), humans (Cibelli *et al.*, 2001), desert Big Horn sheep (Williams *et al.*, 2006), yaks (Li *et al.*, 2007), Siberian tiger (Song *et al.*, 2007), elands (Damiani *et al.*, 2003; Nel-Themaat *et al.*, 2008), red panda (Tao *et al.*, 2009) and Sei whale (Lee *et al.*, 2009). Bovine oocytes but also rabbit ones, have been considered "universal recipient cytoplasm" in cloning research because they represent an abundant resource and

have been demonstrated to be superior in nuclear transfer studies (Dominko, 1999; Zhao ZJ,2006).

The use of the iSCNT technique for cloning has great potential as a tool for the conservation of endangered mammal species, as demonstrated by the successful cloning of gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), banteng (Sansinena et al., 2005), female gray wolf (Kim et al., 2007) and male gray wolf (Oh et al., 2008) The cloned gaur (Bos gaurus) was born after interspecies NT using bovine oocytes but unfortunately died within the first 48 h (Lanza, 2000).

Just a few studies have been carried out on sheep oocytes for iSCNT till now : European mouflon was born after injection in sheep oocytes (Loi P., 2001). In another study, two pregnancies were established after interspecies NT using the domestic sheep (Ovis aries) as recipient cytoplasts and an exotic argali (Ovis ammon) as donor karyoplasts, but both of these pregnancies were lost by 59 day of gestation (White KL, 1999). The goat-sheep cloned embryos have the ability to develop to blastocyst in vitro (Ma LB, 2006), but Zhou H. (2006 and 2008) did not obtain any blastocyst after camel and horse donor cells in sheep oocytes.

The efficiency of embryo cloning by nuclear transfer differs between species and

the procedures used. However, in spite of progresses, the technique SCNT, has presently a very low efficiency rate, thus more studies and approaches are required. Typically, only 1% to 5% of all cloned embryos transferred into surrogate mothers develop into viable offspring (Wilmut I, 2002).

In the whole world 40 species of cervids have been classified and 23 of them are counted among endangered species (IUCN Red List). Nowadays, Fallow deer (*Dama dama*, Linneus 1758) could be considered out of risk but it may be deemed an useful model for Sardinia Red Deer (*Cervus elaphus corsicanus,* Erxleben 1777), living endangered only in Sardinia and Corsica Islands.

In the present study, we compared the ability of somatic cell nuclei of the Fallow deer and Sardinian red deer to dedifferentiate in sheep cytoplasts and to support early development after reconstruction.

Materials and methods

Somatic cell culture

Fibroblast cell culture was generated from an ear biopsy of Sardinian Red deer (*Cervus elaphus corsicanus*) and Fallow deer (*Dama dama*). The skin biopsy was washed in Ca2+- and Mg2+-free Dulbecco's phosphate-buffered saline (DPBS, Gibco) and minced with a surgical blade into 1 mm2 pieces. Minced tissue was plated in a 35 mm (Nunc) culture flask containing 5 ml of TCM 199+ 10% (v/v) FBS, penicillin and streptomycin, 0.1 mM sodium pyruvate at 38.5 ◦C in 5%CO2/air. After 7 to 10 days of incubation, monolayer outgrowths with fibroblast-like morphology at primary culture (PC) were disaggregated with 2.5 mg/ml of trypsin and passaged one to three times (P1–P3) before being frozen (frozen–thawed). The fibroblast were induced to enter a period of quiescence (presumptive G0) by confluence.

Cryopreservation of fibroblast cells

Fibroblast at 90% confluence (1 × 10^6cells/ml), were suspended and frozen in a first generation cryosolution (40%FCS+10%DMSO+50%TCM199)after be disaggregated with 2.5 mg/ml of EDTA-trypsin. Then, fibroblast and cryoprotectant were transferred in a 2ml cryovial precooled at 4°C. The average cooling rate was 2,1°C/min from 0°C to -6O'C and 0.6"C°/min from -60°C to -70°C. -70°C. For thawing, cryotubes were plunged into water at 37°C for 30 to 45 s until half the ice melted, and the content was immediately transferred to a tissue culture tube with 10 mL of TCM199+10%FCS and then centrifuged at 1400 rpm for 5 min.

Oocytes in vitro maturation

Ovaries were harvested from adult females Sarda sheep and transported to laboratory at 30°C in Dulbecco's PBS (Sigma Chemical Co.) supplemented with antibiotics, within 3h after slaughter. Oocyte were collected from ovaries scraping follicoles into Petri dish containing 2 mL of TCM-199 supplemented with Hepes, heparin, streptomicyn and penicyllin. Oocytes collected (COC) were washed in

Hepes TCM-199 (containing 1mg mL^{-1} PVA, 6 IU mL^{1} heparin, 100 IU mL^{-1} penicillin and 100 IU mL^{-1} streptomycin) three times and selected for maturation. Afterward, selected oocytes were incubated in 0,5 ml of IVM medium (TCM-199 with 10%FCS and 24,2 mg mL^{-1} sodium pyruvate, 0,1 mM cysteamyn, 0,05 IU mL^{-1} FSH and 0,05 mL^{-1} LH, 100 IU mL^{-1} penicillin and 100 IU mL^{-1} streptomycin) in 4-well dishes (Nunc, Nunclon, Denmark) under mineral oil. After 24h , cumulus cells were removed by gentle pipeting in H-TCM199 with hyaluronydase. Mature oocytes were selected and were washed three times with SOF with 10% of estrous sheep serum.

Nt with zona

The Nt procedure was carried out by using a micromanipulator (Model MMO-202D; Narishige Instrument, Tokyo, Japan) provided with an inverted microscope (Olympus IX-70, Olympus, Tokyo, Japan) and Hoffman modulation contrast optics. First, completely denuded oocytes were incubated in in Ca21- and Mg21-free modified SOF supplemented with 3 mg/ml BSA, 30 mM NaHCO3, 0.36 mM

pyruvate, 1 mM glutamine,and 50 mg/ml gentamicin (ECM medium), 20 mg/ml Hoechst 33342 and 20 mg/ml cytochalasin B (CCB). After that enuculeation was carried out in handling media (ECM medium, in which NaHCO3 was reduced to 15 mM, and 15mM Hepes was added, with 20 mg/ml of CCB and 2 mg/ml of sucrose (330–340 mOsm, enucleation medium).

Removal of the metaphase spindle was performed with a enucleation pipette (20 micron outer diameter) under fluorescence light. A single fibroblast cell was introduced into the perivitelline space of the enucleated oocyte. Cells and oocytes were fused in a fusion medium (0.3 M mannitol, 0.1mM Mg21, and 0.05 M Ca21). Activation of the fused couplets was performed 2–3 h after fusion by placing the couplets between two electrodes in a fusion chamber containing 3 ml of fusion medium and exposing them to two 60-msec DC pulses of 120 V/mm. Then couplets were incubated in 30-ml drops of SOF medium with 5 mg/ml CCB at 38°C in 5% CO2 for 4 h. The couplets were cultured in SOFaaBSA medium for 9 days.

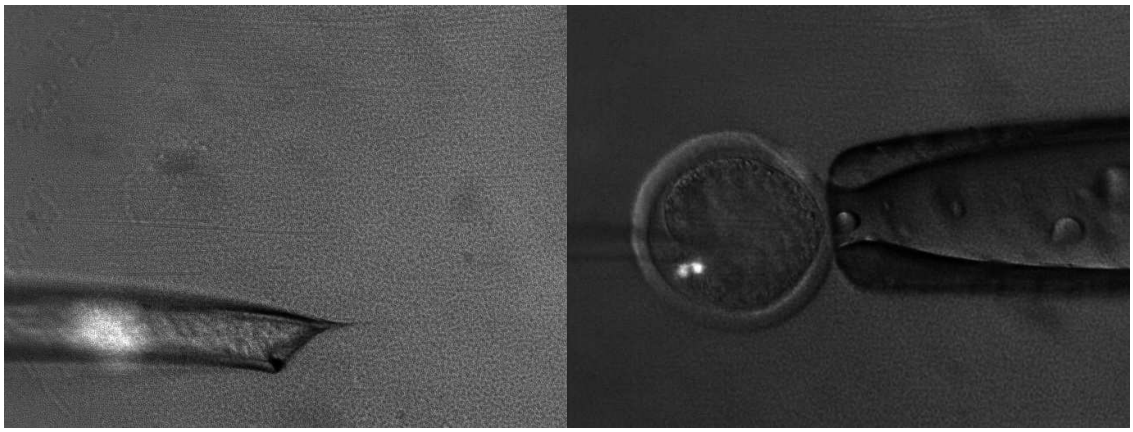

Figure 1:Enucleation of sheep oocyte

Results

In a preliminary experiment, we determined the position of the polar body relative to the metaphase chromosomes. The metaphase plate was adjacent to the polar body in 63% (452/781) of the oocytes of sheep

Fallow deer

In the present experiment, seven fallow deer cell lines were established and used as karyoplast. Two of them were used as fresh colture and five after freeze-thawing. In total, 235 couplets were assembled with a medium fusion rate of 65%,

60,5% using fresh colture, 57,6% with frozen-thawed.

Donor cells derived from the fallow deer skin cells showed fibroblast cell morphology, irrespective of the passage numbers of culture.

Fusion rate

No difference there were between fresh and frozen thawed culture and sheep oocytes fusion rate.

Cleavage rate

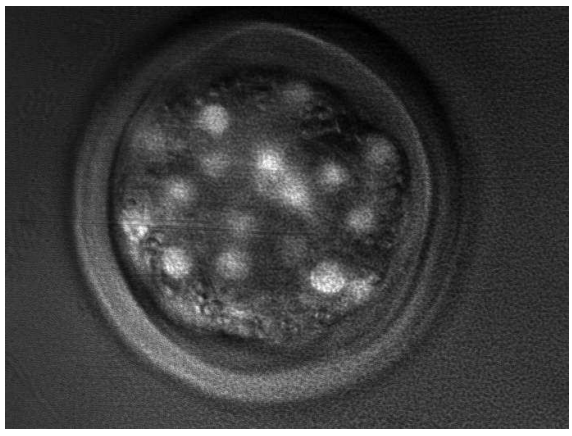

Figure 2:16 cells embryos iSCNT fallow deer/sheep oocyte.

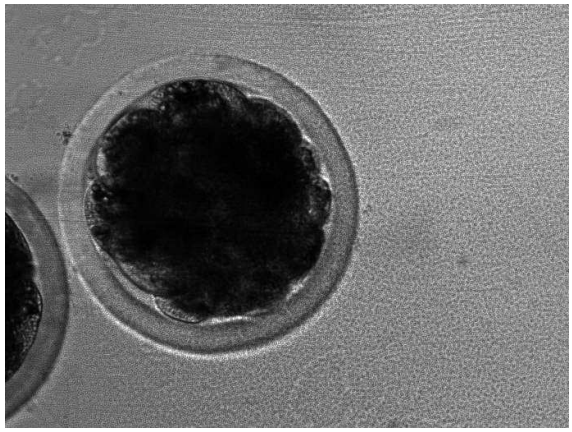

Figure 3:16 cells embryo iSCNT fallow deer/sheep oocyte. Phase-contrast.

The rates of cleavage were different between fresh and frozen thawed fibroblast (p=0,012). No difference (P >0.05) was observed between cleavage of embryo obtained from fresh culture at the first passages (P1-P3) and following (P4-P8), but slightly statistically significant differences (p<0,05) could be observed between frozen thawed colture at the first passages and following. The fallow deer-sheep iSCNT embryos did not develop beyond stage of 16-32 cells

	Fusion rate mean % ± SEM	Cleavage mean % ± SEM	Necrosis mean % ± SEM
Fresh culture **(1-3 passage)**	64±19,29	44,78±5,58[a]	29,8±1,8[a]
Fresh culture **(4-6 passage)**	66,6±6,11	32,41±5,99[a]	23,4±3,3[a]
Frozen-thawed **(1-3 passage)**	55±12,77	19,43±1,77[bd]	43,9±4,1[be]
Frozen-thawed **(4-6 passage)**	61,5±7,78	30,19±0,80[bd]	50,9±7,7[bf]

Table 1: Fusion rate and cleavage in iSCNT embryos using fresh and frozen/thawed cells of fallow deer with sheep oocytes. Necrosis of fresh and frozen/thawed cells of fallow deer. Results are expressed as mean±SEM. Values within the same column with different superscripts (a-b) are significantly different (P < 0.01). c-d are significantly different for (P < 0.05). e-f are significantly different for (P < 0.005).

Red deer

Just one frozen cell line have been used for Sardinian red deer. Fusion rate does not show significant difference between deer and fallow deer such as cleavage rates (p>0,05). Donor cells derived from the deer skin cells showed fibroblast cell morphology, irrespective of the passage numbers of culture. In total, 241 couplets were assembled with a medium fusion rate of 72%.

In our preliminary we obtained sheep morula and blastocyst with SCNT. The deer-

sheep iSCNT embryos did not develop beyond the stage of 8-16 cells stage.

	Fusion mean % ± SEM	Cleavage mean % ± SEM	2cells (%)	4cells (%)	8cells (%)	16cells (%)	32cells (%)
Fallow deer	57,6±10,45	35±9	24%	38%	18%	16%	8%
Red deer	72,12±9,12	37±17	27,6%	29,3%	31%	12%	0

Table2:Fusion rate, cleavage and embryo development in iSCNT embryos using frozen/thawed cells of fallow deer and sardinian red deer with sheep oocytes. Results are expressed as mean±SEM

Discussion

Our results demonstrated that iSCNT embryos from sheep oocytes and fallow deer/Sardinian red deer cells could be produced. The first cleavage indicated that sheep oocyte cytoplasm could successfully initiate the nuclear reprogramming of the transferred Sardinian red deer and fallow deer nuclei and that activation protocol used, induced proper cytokinesis of cytoplasmic division. Ovine MII

oocyte cytoplasm could initiate the reprogramming of a donor nucleus and stimulate the embryonic development: recipient oocytes were enucleated and confirmed by exposure to ultraviolet light. It is, therefore, reasonable to assume that the nucleo-cytoplasmic interaction of presumptive G0/G1 somatic nuclei with enucleated ovine MII oocytes were beneficial for the first mitotic cycles of the reconstructed embryos and the subsequent development to the morula stage. However, none of the reconstructed embryos developed past the morula stage during the culture period, which indicates a compromised developmental ability compared with the parthenogenetically derived ovine embryos (control) and the intra-species nuclear transfer embryos.

This compromised efficiency in the development of reconstructed embryos to the blastocyst stage may be associated with several factors.

The main cause of cloned blastocyst development low efficiency is inappropriate or incomplete ‘‘epigenetic/genomic reprogramming’’ of the transferred nucleus, which partially depends on a variety of technical and biological factors. Furthermore, the incompatibilities between nucleus and ooplasm in interspecies/generic NT study might be associated with abnormal gene

expression that inhibits normal progression of epigenetic events required to create a functional genome. The use of alternative SCNT techniques, such as iSCNT, is an interesting possibility for species with limited availability of oocytes and recipients. However, an 8- to 16-cell in vitro developmental block has been previously reported using this procedure (Yoon et al., 2001; Li et al., 2002; Sansinena et al., 2002, 2003, 2005), particularly when the species of donor karyoplasts and recipient cytoplasts are far apart in their taxonomic classification. Moreover, mitochondrial heteroplasmy is reported to be responsible for the developmental arrest in interspecies/generic reconstructed embryos (Thongphakdee A,2008) possibly from insufficient mitochondrial respiration that hampers the survival of cloned embryos (Dey, 2000). The influence of possible mitochondrial heteroplasmy and/or the deleterious effect of mtDNA of the cloned embryos on development should not be discounted. In the reconstructed SCNT embryos to term, the resultant animals exhibit one of three patterns of mtDNA transmission 1) homoplasmy derived from the recipient oocytes, 2)homoplasmy derived exclusively from the donor somatic cells or 3)heteroplasmy from the fusion partners (St John JC, 2004). In previous reports, the mtDNA of SCNT animals was

exclusively derived from the recipient oocytes (Takeda K, 2006). Conversely, it has been reported that in panda-rabbit iSCNT embryos, mtDNA from both panda fibroblasts and rabbit oocytes coexisted in early blastocysts (Chen DY, 2002). Level of compatibility between nuclear and mitochondrial genomes in iSCNT embryos as the existence of species-specific incompatibility between nuclear and mitochondrial genomes of closely related species has been demonstrated in primates (McKenzie et al. 2003).

Fallow deer and Sardinian red deer are both members of cervidae family. Nevertheless, NT embryos develop differently. Fallow deer/sheep embryo reach 16/32 cells stage, deer/sheep just 8/16. Increased levels of heteroplasmy introduced may explain the decreased cleavage rates in this experiment. The reason for this difference in developmental capacity between the cell lines is unknown but we can hypothized a sort of disparity. The genetic divergence between donor cells may represent a limiting factor influencing iSCNT outcome. In previous studies, remarkable variation exists among telomere lengths in cloned cattle produced from donor cells derived from 4 different kinds of tissue (Miyashita, 2002). Development and competence of the cloned embryos were affected by the

genotype of the donor cells and the cell culture conditions (Heyman,2003).

The transition from maternal to embryonic control of development and embryonic transition occurs by 8-16 cells in sheep. Therefore, the possibility of a relationship between the high rate of development failure and the transition from maternal to embryonic control of development should be considered. The development of interspecies embryos are first manifested at the time when the genome of the somatic cell becomes independent from the maternal transcripts and initiates transcription on its own (Beyhan et al., 2007), the failure of occurrence of which may lead to imposition of developmental arrest (Latham, 2005).

The reasons of such developmental failure in our culture conditions are not clear and it could be ascribed to the quality of our in vitro maturation–in vitro culture (IVC) media or to the NT technique used. Another reason, could be the use of adult fibroblasts as donor cells, which have been reported to give far lower cloning efficiency compared to those obtained with fetal or new born fibroblasts in buffalo (Shah et al., 2009), cow (Saikhun et al., 2002) and pig (Lee et al., 2007).

An aberrant DNA methylation pattern in iSCNT embryos may be another reason. Chen et al. (2004) demonstrated that demethylation of donor repetitive sequences

is driven by the recipient ooplasm, and ooplasms from different species have different capabilities to demethylate genes.

In a preliminary experiment, the rate of fusion was increased with the increase of electric field strength, until these values reach a critical level. The process of cell fusion is controlled by biologically relevant membrane factors among which the rounding of the membrane, the cell shape and the formation of pores are of major influence (Sowers, 1989; Wu et al., 1994; Fuhr et al., 1996; Baumann & Sowers, 1996). In our work, first passages of frozen cells are preferred to improve fusion rate, although primary cells are less synchronous and the cultures have more variability in the cell-cycle analysis than that in cells that have been passaged 6 or 12 times.

In our NT experiments, we used oocytes of similar type and source that were at the same meiotic stage (M-II, no activated). Only 12.0% of the presumptive embryos were fragmented and were unable to remodel the chromatin. From these results, it appears that chromatin remodeling failure may be partially due to the recipient cytoplast, but there are likely other factors involved as well. Such unknown factors may have contributed to the lower rate of chromatin remodeling

in the somatic cell line. Differences in the epigenetic status of the deer somatic nuclei may be a cause of the low rate of chromatin remodeling after NT (Surani A, 2001). A better understanding of the molecular basis for epigenetic variation within the donor cells will help to understand its influence during cell reprogramming.

Cloning is one of several ways of increasing the number of individuals within a population. Clearly, natural breeding is the preferred method for thriving populations; but by definition these are not of conservation concern. However, when populations of free-living species are found to be in decline, conservation biologists begin to seek methods of slowing or reversing the threatening processes. Aim of conservation breeding programmes, with or without the use of assisted reproduction, is the avoidance of inbreeding depression and the associated exposure of rare, and often deleterious, alleles. Some authors affirm that cloning is not a useful method in conservation of endangered animals because genetically identical offspring are undesirable: the objective is to maintain and maximize genetic diversity in the extant population. However, bacteria that reproduce by cloning themselves, show an amazing adaptability.

The potency of epigenetic regulation is exemplified by the finding that queen and worker honeybees are clones. Despite their identical DNA, queens and workers have different behaviors, morphologies and reproductive capacities. They differ because some larvae, the future queens, ingest royal jelly. Effects of royal jelly can largely be mimicked by decreasing the levels of a single repressive epigenetic regulator, the DNA methyltransferase DNMT3 (Kucharski R., 2008)

Genetic resource bank: new approach to biodiversity conservation

Introduction

In the last decade, conservation of cells has seen an intense increase correlated to the establishment of Genome resource banks (GRBs) for the collection and storage of somatic/gonadal tissues, cells, gametes and embryos from genetically valuable individuals of domestic and wild animals. GRB could be an useful tool in the genetic management of captive and wild populations and provides insurance against the sudden loss of diversity in a population (Wildt, 1992).

Although the first GRBs were established in 1952 for domestic animal genetic management programs (Polge and Lovelock 1952: Polge and Rowson 1952), an optimal conservation protocol does not exist. Cryopreservation of DNA kept at -70 °C or -196 °C is widely considered one of the best choices: genetic material could remain substantially intact for thousands of years in a relatively cheap way. However, apart from freezing, there are several other possible methods for the long-term storage of DNA in tissues such as preservation in pure ethanol or freeze drying (Revel A. et al, 2004; Dessauer et al.,1996), as dry smears on FTA Whatman papers (Smith & Burgoyne, 2001) or with the preservative RNA later (Vink et al., 2005). Currently, the preservation of somatic cells may become an option preferable to the often difficult procedure of collecting and preserving

gametes and because they can potentially be used for nuclear transfer or cloning (Ryder & Benirschke, 1997; Wilmut et al., 1997).

Several features need to be considered in a genome resource bank set up of somatic cells:

1. Kinds of the cells;
2. Methods for freezing;
3. Proliferative lifespan.

Fibroblasts isolated from skin tissue are the most frequently cells cryopreserved because they are among the easiest cells to culture in vitro (Freshney I,2002) and to freeze. In spite of this, also cumulus (Bhojwani et al., 2005), granulosa (Wells et al., 1999) and muscle donor cells (Green et al., 2007) have been stored in liquid nitrogen (LN2) for future culture.

Viability of frozen–thawed fibroblasts depends on several variables including cooling/thawing rates and types and concentrations of cryoprotectants that are known to affect structural integrity of plasma membranes (Muldrew et al., 2004).

A commonly used method for freezing fibroblasts consists of adding the cryoprotectant, dimethyl sulphoxide (DMSO, 10% v/v) and freezing the cells in

cryovials at a rate of 1 ◦C/min in a commercial cryo-chamber containing methanol in a low-temperature freezer (LTF; −70 to −80 ◦C) for ≥12 h before storage in LN2 (−196 ◦C; Du *et al.*, 2002; Kragh *et al.*, 2004, 2005). However, bovine fibroblast and cumulus cells have been also frozen in a LTF at a cooling rate of ∼2 ◦C/min, stored at −70 ◦C without storage in LN2 (Kato et al., 2000; Tani et al., 2000).

Cells can be also retrieved from mammalian organs that have been frozen and stored without cryoprotectant for more than a decade (Hoshino, 2009). Moreover, genomic integrity of nuclei may be maintained after freeze-drying (Loi P., 2008), heat-denaturation (Loi P., 2002) and dead (Loi P., 2001) of the somatic cells.

Proliferative lifespan of most somatic cells is limited in vitro (Hayflick et al 1962), and they can enter in a phase of growth arrest and term replicative senescence. Senescent cells remain metabolically active, arrested at the G1/S boundary and lacking the ability to divide (Allsopp R.C.,1996). The characteristics of senescence include alterations in morphology (increased cell surface and volume, flattening of the cell), function (over-expression of cytokines), gene expression patterns,

resistance to apoptosis, shortened telomere length (Campisi 1996) and, above all, chromosome alterations (Giraldo A.M. 2006). A number of known cytological mechanisms can account for the formation of abnormal chromosome number and morphology in cell strains. Cytokinesis failure, mitotic irregularities, and abnormal chromosome segregation can all result in abnormal karyotypes (Levan and Hauschka, 1953; Ford et al., 1959; Chu, 1962). Stable diploidy was maintained in rat cell cultures (Hsu and Kellogg, 1960a,b), but mouse cells begin to change their chromosomal constitution even in primary cultures (Chu, 1962).

In our work, we would observe how freezing cervids (*Dama dama*, Linneus 1758) fibroblasts in a LTF (−80 ◦C) affects viability, apoptosis and ploidy status and if could be feasible to set up a genetic resource bank and for SCNT.

Material and methods

Fibroblast culture

Fibroblast cell culture was generated from an ear biopsy of Fallow deer (*Dama dama*, Linneus 1758). The skin biopsy was washed in Ca2+ and Mg2+ free

Dulbecco's phosphate-buffered saline (DPBS, Gibco) and minced with a surgical blade into 1 mm^2 pieces. Minced tissue was plated in a 35 mm (Nunc) culture flask containing 5 ml of TCM 199+ 20% (v/v) FBS, penicillin and streptomycin, 0.1 mM sodium pyruvate at 38.5 ◦C in 5%CO2/air. After 7 to 10 days of incubation, monolayer outgrowths with fibroblast-like morphology at primary culture (PC) were disaggregated with 2.5 mg/ml of EDTA-trypsin and passaged one to eight times (P1–P8) before being frozen.

Cryopreservation of fibroblast cells

Fibroblast at 90% confluence (1 × 10^6cells/ml), were suspended and frozen in a first generation cryosolution (40%FCS+10%DMSO+50%TCM199)after be disaggregated with 2.5 mg/ml of EDTA-trypsin. Then, fibroblast and cryoprotectant were transferred in a 2ml cryovial precooled at 4°C. The average cooling rate was 2,1°C/min from 0°C to -6O'C and 0.6"C°/min from -60°C to -70°C. -70°C. For thawing, cryotubes were plunged into water at 37°C for 30 to 45 s until half the ice melted, and the content was immediately transferred to a tissue

culture tube with 10 mL of TCM199+10%FCS and then centrifuged at 1400 rpm for 5 min.

Evaluation of necrosis

Cell necrosis was detected by propidium iodide (PI) labeling of fibroblast cultures with increasing passage number. Cells that stained with red, propidium iodide, were considered necrotic. Live, viable cells had little or no fluorescence.

Chromosome analysis

Fresh and frozen–thawed fibroblasts were cultured in tissue culture 4 Well Dishes (Nunc) until reaching 50% confluence. According to modified Iwasaki method (1992), cells were incubated in TCM199medium containing 0.28 µg/ml colcemid (Gibco) overnight. After that, fibroblasts were disaggregated with 2.5 mg/ml of trypsin, re-suspended in TCM199 with 15% FBS and centrifuged at 1000 g for 5 min. After discarding supernatant, pellets were re-suspended in 5 ml of 0.075 M KCl (hypotonic solution) and incubated at 38 ◦C for 5 min. Cells were

then centrifuged (1000g for 5 min), fixed for 10 min in methanol and acetic acid (3:1) and centrifuged again at 1000 g for 10 min. The fixation/centrifugation step was repeated three times. Then, fibroblasts were placed on ice-cold slides and dried in a gas flame. Fibroblast chromosomes were stained with a freshly prepared solution of 0.4% Giemsa for 10 min (Gomez *et al.*, 2006). Chromosome spreads were examined by brightfield microscopy at ×100 magnification to assess the chromosome composition of each fibroblast culture. Images were captured using a digital camera (Moticam 2000, Motic China Group Co., Ltd) and analysed with Image J software (free version). Chromosome compositions were categorized as: either diploid cells containing a normal set of chromosomes (n=68), hypoploid ($n \leq 68$) or hyperploid ($n \geq 68$) cells, containing less than or more than the normal diploid number of chromosomes, respectively. Chromosome spreads that were nonsynchronized, scattered or overlapped were excluded from analysis.

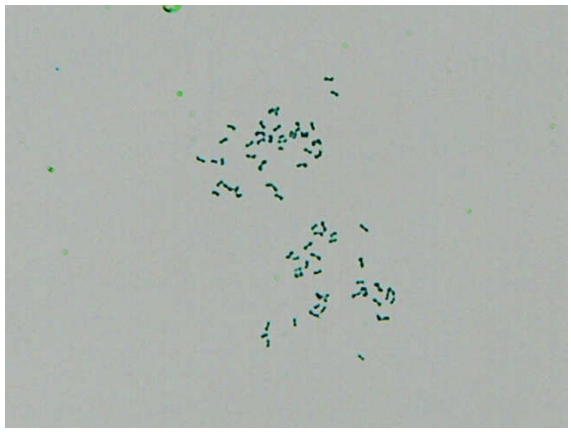

Figura 4 Hypoplody in fallow deer fibroblast

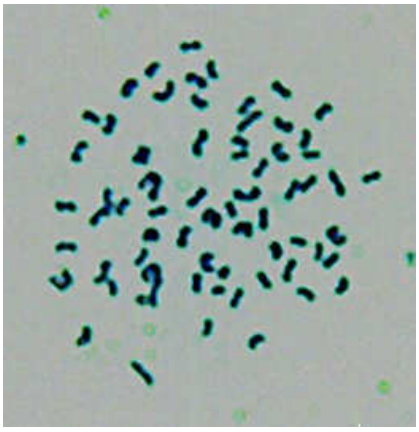

Figure 5 Normal ploidy in fallow deer fibrobast

Sta tistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). All the data were checked by the Kolmogorov-Smirnov's test in order to evaluate their normal distribution. One-way ANOVA was performed in order to compare means between groups. Tukey post-hoc test was applied in case of homogeneity of variance (Levene's test), otherwise Dunnet-T3 post-hoc test. Pearson correlation was used to evaluate correlations

Results

Fibroblasts were in culture for 7-8 days before reaching 100% of confluence at P1 for fresh colture, 9-10 days for frozen–thawed. On the other hand, following passages requested from 5-6 (fresh) to 6-7 (post thawed) days to reach full confluence.

1440 fallow deer fibroblast cells were analyzed. The most frequent chromosomal abnormality was hypoploidy in fresh and post thawed colture (129/360,35,8%,

301/900, 33,4% respectively, not statistically different), and it occurred more frequently than hyperploidy (p≤0,001).

Fresh culture

Passage	**1**	**2**	**3**	**4**	**5**	**8**
Hyperplody(%)	8,33	5,00	6,67	5,00	3,33	0,00
Hypoploidy(%)	15,00^{ab}	21,67	28,33^{b}	31,67^{b}	40,00^{d}	50,00^{d}

Table3 Hyperploidy and hypoploidy in fresh culture ab p< 0,05; cd p<0,0001

Frozen-thawed culture

Passage	**1**	**2**	**3**	**4**	**5**	**8**
Hyperploidy(%)	7,33	5,33	6,00	5,33	4,67	2,00
Hypoploidy(%)	15,33^{ac}	18,67	28,00^{b}	28,00^{b}	34,00^{d}	46,00^{d}

Table 4 Hyperploidy and hypoploidy in frozen thawed culture ab p<0,05;cd p<0,0001

Hypoplody cell percentage raises from passage 1 to passage 3 ($p \leq 0,05$) and became more pronounced after passage 5 ($p \leq 0,0001$). On the other hand, there are not differences in hyperploidy among passage in both, fresh and cryopreserved culture.

Frozen culture showed high percentage of necrosis at first passage (39,13%) compared ($p<0,0001$) to fresh ones (22,3%). In spite of this, results showed a significant increase in fluorescence just from high (P5-8) passages $p<0,0001$ in thawed culture, while in fresh culture, necrosis increased in passage 3 ($p<0,0001$).

Necrosis and hypoploidy are strictly correlated ($p<0,0001$) in fresh and post thawed culture.

Discussion

The necessity for genome resource banking of valuable individuals, breeds or species follows the dramatic increase in the loss of natural habitats, high rates of

inbreeding, and reduced reproductive performance in both wild and domestic animals.

The results of the present study indicated that in vitro culture affect chromosomal status and necrosis of fallow deer fibroblast cells. LTF-80°C freeze effects seem to bear on necrosis but not on ploidy as already showed for LN2 in previous works(ref).

Hypoploidy seem to be the most common chromosomal abnormality observed. Currently it is not clear the exact cause of the hypoploidy but abnormal chromosomal composition of tissues in primary cultures or during following passages is higher than in *in vivo* tissues (Chu, 1962) and this suggests an imperfect culture of the cells. The cell lines analyzed in this study contained an increased percentage of aneuploid cells immediately after the primary culture. This indicates that alterations in the cytokinetics, DNA replication, and/or condensation may occur during the establishment of the primary culture.

Most hypoploid abnormalities are caused by failure of sister chromatids in mitosis or paired chromosomes in meiosis to migrate to opposite poles during cell division (Ono, 1971). Instead hyperploidy, may be a result of cytokinetic

failure, including endo replication, non-disjunction(Levan and Hauschka, 1953; King et al., 1988, 1990).

Cytokinesis failure, mitotic irregularities, and abnormal chromosome segregation can all result in abnormal karyotypes (Levan and Hauschka, 1953; Ford et al., 1959; Chu, 1962). An additional proposed mechanism is abnormal phosphorylation of the histone H3 at the serine 10 during metaphase leading to abnormal chromosome condensation and chromosome loss during mitosis (Wei et al., 1999; Ota et al., 2002; Dossabhoy et al., 2003).

It is well know that even i*n vivo*, each nuclear gene is subject to a small risk of spontaneous mutation at an estimated rate of 10^6 mutations per gene per cell division (Oback and Wells, 2002). It fallows that in vitro chromosome aneuploidy (Denning et al.,2001) may reach 85%, as reported in porcine fibroblast after prolonged culture *in vitro.* On the other hand, in fetal porcine, sheep, and bovine fibroblasts aneuplody remained low after prolonged culture *in vitro* (5%, 13%, 20%, respectively; Zhu et al., 2004; Denning et al., 2001; Kubota et al., 2000)suggesting that, not only environmental factors such as culture conditions

and seeding density but also as donor age could be involved (Campisi 1996, Balin 2002, Zhu 2004).

Moreover we can not exclude that high concentration (0.28 _g/mL) of and extended exposure time to Colcemid would be correlated to aneuplody (Gomez MC, 2008).

The lifespan of somatic cells is affected by culture conditions (Falanga and Kirsner, 1993), age of the donor animal (Kasinathan et al., 2001), tissue and species of

origin (Rubin, 1997). Kasinathan et al. (2001) estimated that fetal bovine fibroblasts divided 30 times before reaching senescence; however, variations in the proliferative

capacity can occur due to slight changes in the culture conditions. Time in culture induces changes in post-translational modifications of histoneH3(lysine 18),H4(lysine 8), and H1 (Enright et al., 2003). It is also possible that in vitro culture conditions induce changes in the level of phosphorylated histone H3 resulting in abnormal

chromatin structure, missegregation, and loss of chromosomes.

In embryos several explanations have been mentioned: aging of oocytes (Maudlin and Frazer, 1978; Ho et al, 1994), the IVF system itself (Frazer et al,1976; Maudlin and Frazer, 1978; Iwasaki and Nakahara, 1990a), and the percentage of morphologically normal spermatozoa (Ho et al, 1994) but they can not be adopted for fibroblasts.

Moreover several authors reported that theoretically, the process of non-disjunction should produce an equal number of hypo- and hyper haploid . However in many cytogenetic studies a significant excess of hypohaploid complements is always reported. Thus, hypohaploid chromosome sets might be a technical artifact and hyperhaploid spreads are considered as evidence for non disjunction(Martin ,1984).

We can state, from our experience, that the most importance aspect of the culture is a good initial concentration of seeding and an adequate composition of media. In preliminary observations, we found that culture with low seeding concentration (more than 10 days to reach confluence) showed more anomalies than high –concentration culture even in primary culture. On the other hand,

using of trypsin or pronase for dissociating and TCM199 or DMEM for culturing, did not affect our culture.

In conclusion, cells frozen at LTF-80°C could be used as genetic banks and source for somatic cell nuclear transfer, but only provided that could be improved culture conditions and used earlier passages (until passage 4). Moreover it could be suggested a screening of the donor cells and cell lines.

Fallow deer in vitro embryos production from *in vivo* and post mortem gametes collection

Introduction

Post-mortem collection of gametes from endangered animals and free living populations could serves as a valuable source to understand the physiology of the species. Moreover, gametes recovery could be useful to develop species-specific protocols for *in vitro* production of embryos (IVP) (Raphael et al. 1991; Loskutoff et al. 1995; Hall-Woods et al. 1999; Keller et al. 1999; Herrick et al. 2004; Roldan et al. 2005; Rao et al. 2010) and somatic cell nuclear transfer technology (SCNT) to conserve endangered species. A few attempts have been made to explore the feasibility of recovery, in vitro maturation (IVM) and fertilization (IVF) of oocytes collected after accidental death as in kudu and impala (Loskutoff et al. 1995), blesbok (Winger et al. 1997; Brad et al. 2004), addax antelope (Hall-Woods et al. 1999), blackbuck (Keller et al. 1999; Rao et al. 2010), black wildebeest (Brad et al. 2004), dorcas gazelle (Roldan et al. 2005) and springbok (Brad et al. 2004; Herrick et al. 2004; Krisher et al. 2006). In recent decades, concern about nature conservation has grown rapidly as a consequence of accelerating rates of

natural habitat loss, habitat fragmentation, degradation, and resulting extinctions of species (Lloyd J., 2009). Assisted reproductive techniques (ART) and Genetic Rescue Bank (GRB) could be considered useful tools to cope with fragmentation of habitat, one of the main problem in conservation and preservation of genetic variability (Zachos F.E., 2011). ART's application allows *in vitro* production of embryos from genetically valuable individuals, with potentially more rapid expansion of populations than natural breeding. Moreover, conservation of genetic resource in Genetic Rescue Bank preserves biodiversity and gives future reproductive opportunities (Leon Quinto T., 2008).

Reproductive Physiology Cervidae

Reproductive strategies of Sardinian red deer and fallow deer could be included among so-called "K strategists". They have lesser reproductive potential, lower fecundity (i.e. singleton births) and later sexual maturation (>1 year) and are generally mixed grazer/browsers with marked gregariousness and social/behavioural plasticity. Both are short-day breeder that mates during a rut

occurring shortly after the autumn equinox.

Males and females reach sexual maturity at 18 months (Beccu E., 1989). Hinds are seasonally polyoestrous, and exhibit an oestrous cycle varying from 19 to 20 days. Non-pregnant animals are capable of exhibiting continuously repeated oestrous cycles. In non-pregnant females the onset and termination of oestrous cyclicity occur in autumn and spring respectively, and they can express 5–8 cycles. Anoestrus is characterized by low peripheral plasma concentrations of progesterone indicative of complete ovulatory arrest, and may persist for 4–6 months from spring to early autumn.

Photoperiod is the primary environmental cue controlling seasonal breeding in deer. The transition into the female breeding season is characterized by 'silent ovulations' (ovulations not preceded by overt oestrus) and short-lived (8–10 days) corpora lutea. In fallow deer (*Dama dama*), multiple successive silent ovulations leading up to the start of the breeding season have been observed (Asher, 1985).

Luteinisation of post-ovulatory follicles is associated with increased secretion of progesterone, with maximal concentrations in peripheral blood occurring

between days 10 and 16 of the oestrous cycle (Day 0 = oestrus). Luteal regression, which for fallow deer involves the classic interaction between oxytocin and prostaglandin F2a (Asher et al., 1988a), occurs rapidly about 1–3 days before the return

to basal progesterone concentrations and return estrus. In the seasonally breeding red deer hind, conception rates following natural mating during the autumn rut appear to be high (>85% per mated estrus), and early embryonic mortality (i.e. within 20 days of fertilization) appears to be low (i.e. <1%; Berg et al., 1994). Given that hinds are polyestrous, under circumstances where nutrition and disease are not limiting, adult female red deer generally exhibit greater annual fertility (i.e. >96% pregnancy rates), albeit with lesser (i.e. <1% of pregnancies) twinning (Asher and Pearse, 2002) e red deer embryo enters the uterus 2–3 days later at the blastocyst stage, 5–6.5 days after ovulation. Embryonic implantation in red deer occurs shortly before day 27 from ovulation (McMahon et al., 1997). Luteal support of pregnancy in red deer persist through the term of gestation, with peripheral blood progesterone concentrations remaining elevated to within 1–2 days of parturition the presence of a functional

corpus luteum is a requisite for a viable pregnancy (Asher et al., 1996; Plotka et al1982). Conception was highly synchronized (Pintus E. 2008) and gestation lengths for 230-240 days.

Fallow deer IVP

'Cervidae' family is represented by over 40 species and 200 subspecies (Whitehead, 1993) in the whole world. It is well known that more than half of the species (or subspecies) in this family are threatened or endangered. Physiology, reproductive strategies and consequently, in vitro embryo production (IVP) vary widely within and between regions and species for their wide set distribution.

Oocytes maturation, fertilization and early embryonic development *in vitro* have recently been studied for Iberian red deer(*Cervus elaphus hispanicus*, García-Alvarez O., 2011), white-tailed deer*(Odocoileus virginianus*-Siriaroonrat B., 2010), red deer(*Cervus elaphus*-Berg et al., 2008), sika deer(*Cervus nippon*-Comizzoli P., 2001). So far, to our knowledge, no studies have been carried out on fallow deer(*Dama dama,* Linneus 1758) oocytes collection, fertilization and culture.

Fallow deer are dimorphic ungulates with typically polygamous mating system and high stagionality(Clutton-Brock et al. 1988; Apollonio et al. 1992; Moore et al. 1995): reproductive behavior may be observed between late August and December. However, most matings occur during the rut from mid-October until the beginning of November(McElligott et al. 1998, 1999).

The first aim of our study was to explore for a good protocol to collect gametes from free living population of fallow deer during breeding and no breeding season. Moreover, we evaluated if collection of oocytes from free living fallow deer, accidentally died, could be feasible for in vitro production of embryos.

Materials and Methods

Reagents

All chemicals used in the present study were purchased from Sigma Chemical Co. (Madrid, Spain), unless otherwise indicated.

In vitro maturation

Ovaries were obtained from dead free living animals during breeding and no breeding season. We consider oocytes collected from January to July in "no breeding season", from August to September in "prebreeding", from October to November breeding and December "post breeding" in relation to follicle appearance and animal behaviour. "Out of breeding" season includes the period from December to September.

Ovaries were rescued within 3 h after death of animal and transported to the laboratory at 20-25°C (room temperature, RT) in pairs in screw-top plastic tubes containing Dulbecco's PBS solution. Immature oocytes were collected from ovaries scraping follicles into Petri dish containing Hepes-TCM-199 (1mg mL-1 PVA, 6 IU mL1 heparin, 100 IU mL -1 penicillin and 100 IU mL -1 streptomycin). Oocytes that were degenerated and/or with expanded cumulus cells were removed. Those with dark homogeneous cytoplasm and surrounded by packed cumulus cells were selected and randomly placed in four-well plates containing 500 µL of TCM 199

with 10%FCS and 24,2 mg mL -1 sodium pyruvate, 0,1 mM cysteamyn, 0,05 IU mL -1 FSH and 0,05 mL -1 LH, 100 IU mL -1 penicillin and 100 IU mL -1 streptomycin) in 4-well dishes (Nunc, Nunclon, Denmark) under mineral oil for 24h.

Meiosis progression

At the end of the in vitro maturation period, COCs were examined for cumulus cell expansion and, after cumulus remotion, for extrusion of the first polar body.

Meiosis status was evaluated after in vitro fertilization. Oocytes no cleaved after 48 h.p.i., were stained with Hoechst 33342 and observed in a fluorescence microscope (Olympus N70) to determine maturation status and/or fertilization by the presence of ≥2 pronuclei or decondensed sperm heads. Incidence of nuclear maturation was defined as the number of oocytes at the telophase I/MII stage relative to the total number of oocytes cultured in vitro (Egerszegi I, 2007).

Electroejaculation

Semen was collected from 4 adult stags lived in the same enclosure during the breeding season (end of September). Stags were teleanesthetized by an intramuscolary injection (Wiener mischung) of xylazine (Rompun®, SS, Bayer) and tiletamine-zolazepam (Zoletil 100®, Virbac). Doses were: xylazine 1,25 mg/Kg and tiletamine-zolazepam 1,25 mg/kg in Sardinian red deer and xilazine 1,85 mg/kg and tiletamine-zolazepam 1,85 mg/kg in fallow deer. Rectum and prepucial area were cleaned with physiological saline solution before electroejaculation. Electroejaculation was carried out using a rectal probe measuring 375 mm length and 200 mm width connected to a power source which allowed to control voltage and amperage. Electric impulses progressively increased were applied starting from 0,5 V till maximum 8 V. Each impulse last 5s followed by 5s pause. Ejaculation usually occurred between 2-4 V in fallow deer. Samples suspected of urine contamination (abnormal color and observations during the electroejaculation procedure) were discarded. However, collection of semen in fraction allow to avoid bulbo-urethral fraction and urine contaminations. Semen collected was

immediately diluted in 1:1 (v/v) in TCF with egg yolk (20%) and transported at ≤20°C to the laboratory where all analysis were carried out. Only sperm-rich fraction of each ejaculate was used for freezing. Semen was diluted until reaching $200*10^6$ spermatozoa/ml with 6% glycerol. Semen was packed in 0.25 mL straws (IMV, L' Aigle Cedex, France) and frozen in nitrogen vapours (4 cm above liquid nitrogen) for 10 min, and then transferred to liquid nitrogen. Straws were thawed in water bath at 37 °C for 35". CASA SYSTEM V.12 was used to perform analysis post thawing.

In vitro fertilization

After 24 h, cumulus cells were removed by gentle pipetting in H-TCM199 with hyaluronydase. Mature oocytes were selected and washed three times with SOF with 20% of estrous sheep serum (Berg DK, 2002).

Then, the oocytes were transferred into 4-well dishes containing 450 µL of synthetic oviduct fluid as described by Berg and Asher for red deer (IVF-SOF) with 20% ESS.

Frozen electroejaculated semen from adult fallow deer was used for IVF. Spermatozoa in 0.25 ml pellets were thawed at 37 °C for 30 s, transferred to a glass tube with 1 ml SOF medium, and incubated for 40 min at 38.5 °C under 5% CO_2 in air. Only the upper 0, 6 ml was collected, evaluated and used for fertilization.

Semen and selected oocytes were co incubated in four well with 0,45 ml of IVF-SOF till 18 h at 38.5 °C in 5% CO^2, 5% O^2 and 90% N^2 in humidified atmosphere.

In vitro culture

Presumptive zygotes were washed three times in SOFaaBSA (Locatelli Y, 2005) and cultured in the same medium in 5% CO^2, 5% O^2 and 90% N^2 humidified atmosphere for 9 days. Cleavage rate was evaluated at 24h and 48h post insemination (hpi).

Statistical analyses

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS Inc, Chicago, IL, USA). For each variable means ± standard deviation were calculated. All the data were checked by the Kolmogorov-Smirnov's test in order to evaluate their normal distribution. One-way ANOVA was performed in order to compare means between groups. Tukey post-hoc test was applied in case of homogeneity of variance (Levene's test), otherwise Dunnet-T3 post-hoc test. The level of significance was $p< 0,05$.

Results

Semen

Median value of electro ejaculated semen parameters before (fresh semen), after thawing (P-th) and after 2h (2h p-th) at 37°C are shown in the table.

	Fresh Medium value	±	DS	P-th Medium value	±	DS	2h p-th Medium value	±	DS
Motile	71,21	±	16,75	49,67	±	1,63	24,78	±	2,06
Progressive	29,75	±	13,23	12,00	±	0,72	5,00	±	0,54
VAP	75,03	±	29,87	40,92	±	2,35	29,52	±	1,53
VSL	61,13	±	25,02	29,22	±	0,38	22,01	±	1,96
VCL	119,47	±	39,86	77,44	±	5,88	56,74	±	5,09
ALH	7,11	±	2,74	8,08	±	0,13	5,78	±	0,48
BCF	22,31	±	16,46	14,10	±	0,57	12,71	±	1,36
STR	77,71	±	10,88	70,56	±	3,03	71,33	±	3,86
LIN	52,00	±	10,97	41,22	±	2,99	40,67	±	5,17
Rapid	51,79	±	28,09	29,56	±	3,51	8,89	±	0,42
Medium	19,04	±	11,28	20,00	±	3,40	15,67	±	1,78
Slow	19,71	±	12,02	43,44	±	4,42	43,78	±	7,51
Static	9,08	±	7,62	7,00	±	2,62	31,56	±	9,43

Table 5Electroejaculated sperm parameters: median value

Oocyte harvesting, fertilization and culture

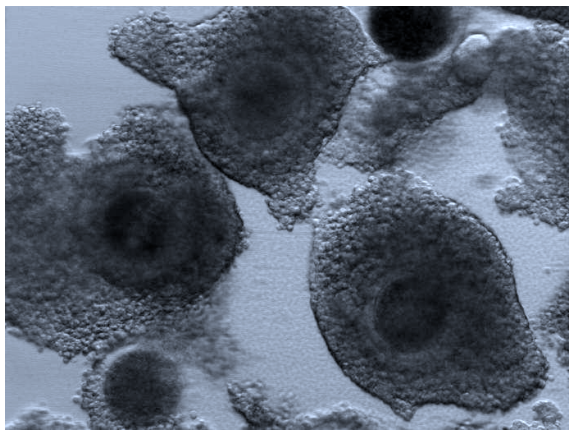

Figure 6 Cumulus expansion after 24h in vitro maturation

A total of 144 cumulus-surrounded oocytes were selected for in vitro maturation.

As showed in the table (Table 1), the number of oocytes collected from each ovary does not differ significantly between breeding and no breeding season.

	Oocytes collected	**Oocytes collected /ovary**	**Maturation rate (%)**	**Cleaved (%)**
No breeding	31	8,5±1,88	65,67[a]	20,36[c]
Prereproductive Seasons	32	10±3,04	70,97[a]	22,71[c]
Reproductive Seasons	67	8,25±1,73	78,13[b]	52,34[d]
Postreproductive Seasons	14	7±0	71,43[a]	10,00[c]

Table 6Within columns, values significantly differ for p<0,05 a-b, p<0,01 c-d

Maturation rate showed slightly statistical difference between breeding and out of breeding season (p<0,01).

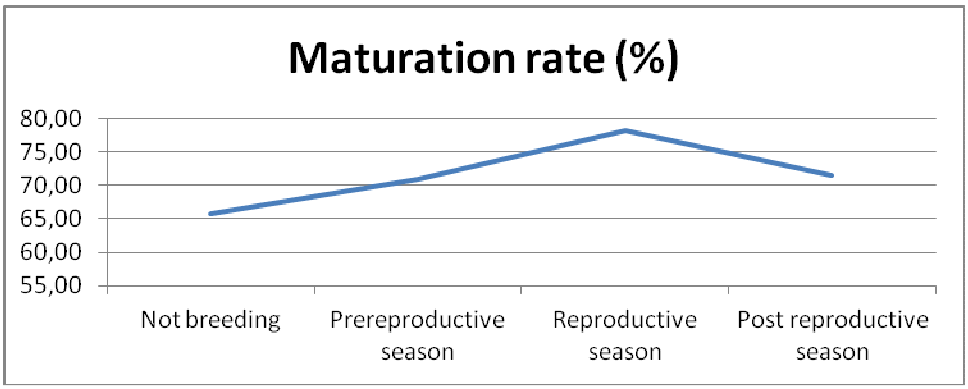

Table 7 Maturation rate during breeding and no breeding season

On the other hand, cleavage was higher during breeding season ($p<0,05$).

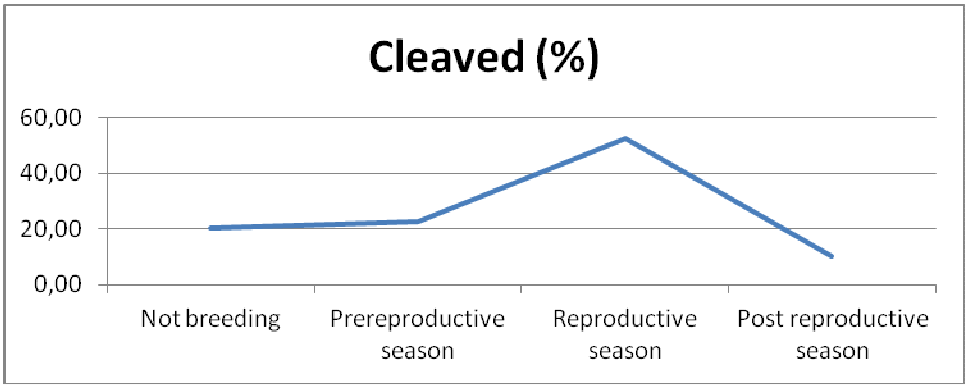

Tabella 8 Fertilization trend during breeding and no breeding season

Embryos cleaved in vitro up to the 8-16 cells stage but none progressed to the blastocyst stage.

16 cells	8 cells	4 cells	2 cells
47,06%	29,41%	17,65%	0,06%

Table 9 Cleavage 48 h.p.i

Discussion

This study represents the first attempt to generate fallow deer embryos *in vitro*. Unusually, the collection has been carried during and out breeding season in free living population.

A common problem when dealing with free living endangered species is the limited number of samples. The hunting of endangered species is not allowed and oocytes could be collected only from animals dead accidentally. Gametes recovered from post-mortem ovaries or testicles have been considered as valuable resource for conservation of germplasm of wild or endangered animals. Several papers describe optimal in vitro protocol assessed for farm cervids as model for endangered species. This approach could be of limited use in endangered species: endangered animals are not bred, the collection is not

concentrated in a season and animals sometimes could not be in best physical and reproductive conditions. We noticed that during breeding season could be possible to produce a fairly good percentage of embryos. Nevertheless, in regard to genetic value, it could be useful the collection during the whole year. Locatelli Y. (2006) with OPU obtained high cleavage rates independently of season or culture conditions ($P > 0.05$) but after 8 days of culture in SOFaa–BSA medium, embryos remained blocked at the eight-cell stage and no blastocysts were produced.

The percentage of fertilization in vitro could be compared to recent publications on red deer (63%- Locatelli Y., 2005) or Iberian red deer (50%, García-Alvarez O., 2010) if we consider only oocytes collected during breeding season. Locatelli noticed just a tendency ($P = 0.26$) for a higher ability of oocytes to develop to the blastocyst stage during breeding season. According to Berg D.K. (2003) early embryo cleavage rates decreased during no breeding season, blastocyst development rate dramatically decreased, with no blastocyst development after July.

Fallow deer oocytes followed a similar time course to complete the MII stage as has been reported for other domestic ruminant species (Moor and Crosby, 1985; Sirard et al., 1989; Pawshe et al., 1994) and cervids (Berg DK, 2002). Culturing red deer oocytes for 24 and 27 h allowed 50 and 75% of the oocytes to develop to MII when using standard ruminant oocytes maturation techniques (Berg DK, 2002). Nuclear maturation rates have ranged from 61 to 78% in red deer (Berg D.K., 2002, Comizzoli P., 2001), sika deer (Comizzoli ,2001), reindeer (Krogenaes A, 1994). Maturation rate differ significantly between reproductive season and out of breeding period but not such as cleavage rate. Then, fertilization is not only correlated with maturation. Probably during breeding season, oocyte acquired specific competence. We hypothesized that exogenous gonadotrophin administration is not sufficient to fully restore oocytes function during the non-breeding season in strongly seasonal free living animals. Moreover should be considered that low maturation rates in summer could be correlated to time interval between animal death and collection of ovaries because the onset of autolytic changes that cause degenerative changes in the follicles. In previous studies on ovaries from free living animal maturation rates is variable: eland

antelope (63%), springbok (35%), blesbok (39%), dorcas gazelle (47%) black wildebeest (52%).

We did not notice statistical significantly differences in the mean recovery of oocytes for ovary. On the other hand, in OPU in red deer, mean number of follicles aspirated and COCs recovered per hind per session decreased significantly during the non-breeding season ($P < 0.001$) (Locatelli Y, 2006). Probably we did not notice any difference because animals did not belong to a unique age class, have different body conditions, and above all dominance status. However, although the differences are not statistically relevant, we noticed a tendency towards rise during prebreeding season.

Serum, homologous and heterologous seem to have a beneficial effect on sperm motility (Bavister 1975; Brown and Senger, 1980; Mandal et al., 1989). We used 20% FCS in SOF media as capacitating agent as previously described in red deer (Berg D.K. 2003). We have never used deer serum but in previous paper seemed to significantly decrease fertilization rates from 73 to 24% (Berg D.K., 2003).

Unfortunately we could not surgically cannulated oviducts of fallow deer to evaluate exact oviductal fluid compositions but we hypothesized that could be

more similar to red deer than sheep or bovine ones. When fallow deer zygotes were cultured in vitro, there was cleavage up to the 8- to 16-cells stage but none of the embryos progressed to the morula or blastocyst stage, suggesting the existence of a developmental block. One possible explanation is that embryo culture conditions and compositions of media were not adequate for fallow deer embryos. Moreover we should consider that time of oocyte rescue was not fixed: Miao et al. (2007) demonstrated that the blastocyst rates declined remarkably when the time interval between removal of ovaries and animal death increased. Developmental competence is affected by several factors such as age, season, reproductive status, nutrition and health of the donor animal (Ptak et al. 1999; Saito et al. 2001; Zheng et al. 2001; Izquierdo et al. 2002; Koeman et al. 2003). In vitro embryo culture procedures for wild ungulates are currently limited to protocols developed for domestic animals and Berg D.K. (2003) highlight cervine species-specific in vitro fertilization and culture requirements, even at a subspecies level. Using such protocols, blastocysts have been obtained after oocyte in vitro maturation, fertilization and culture in the axis deer (Axis axis), red deer (Comizzoli, 2001; Berg DK, 1995) and sika deer (Comizzoli 2001).

Post-mortem-derived oocytes that were matured in vitro developed to morulae/ blastocysts following IVF in klipspringer (Raphael et al. 1991), kudu and impala (Loskutoff et al. 1995), blackbuck (Keller et al. 1999) and springbok (Krisher et al. 2006) antelopes.

Is heterologous IVF a true test to assess in vitro fertility in fallow deer (*Dama dama*)?

Introduction

The best source of germplasm in species can not be hunted is constitutes from postmortem collection and electroejaculation. Assessment of the sperm quality by the evaluation of standard semen parameters is a routine procedure in the prediction of male fertility. However, the best assessment of in vitro function involves sperm–oocyte interaction (Bavister 1990). In vitro homologous fertilization is the most adequate method to assess the fertility. Some authors have used homologous IVF assays as a predictor of fertility using zona-intact oocytes. Other authors, have used zona-intact oocytes of domestic animals to evaluate spermatozoa functionality by heterologous IVF tests (Roth TL,1998 and 1999). Heterologous in vitro fertilization (IVF) is an attractive method to evaluate the fertilizing capacity of sperm samples in wild species because it does not require the use of valuable homologous gametes, above all when oocytes are poor available. The cross-species fertilization of oocytes from domestic farm species has been proven successful, using cryopreserved sperm form deer (Comizzoli et

al. 2001) and non-domestic Bovidae (McHugh and Rutledge 1998; Roth et al. 1999. Recently, the sperm function was evaluated in several endangered African antelope by means of a heterologous IVF system with zona-intact domestic cow oocytes. Moreover, heterologous IVF has been used to evaluate fertility to assess the fertility of thawed ram sperm samples using calf oocytes with zona pellucida (Garcia Alvarez O., 2010).

Usually, in heterologous IVF cleavage rate, fertilization rate, penetration rate, binding have been evaluated. (Kouba et al. 2001). In fact, the fertilizing capability of spermatozoa is believed to depend largely on their capability to cross the zona pellucida (D. Waberski et al). Three different systems have been developed to measure the capability of spermatozoa to interact with the ZP: (1) sperm–zona binding tests; (2) sperm–zona penetration tests; and (3) in vitro fertilization (IVF) tests. Binding assays depend on surface molecules in spermatozoa and their binding to sites or receptor-like molecules in the ZP, typically with epitopes on a protein functionally analogous to ZP2 in the mouse zona (Yanagimachi, 1994).

Our aim was to evaluate correlation among cleavage, fertilization, penetration rate and binding with cleavage in homologous IVF. We would like to understand which parameter in heterologous fertilization could be evaluated to predict fertility in homologous IVF.

Material and methods

All animal experiments were performed in accordance with DPR 27/1/1992 (the Animal Protection Regulations of Italy) which conform to European Community regulation 86/609, and which adhere to guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction.

Oocyte maturation

Adult (4–6 years old) ovine ovaries (Sarda sheep) were collected from slaughterhouse. After remotion, the reproductive tract was excised, and ovaries were placed in pairs in screw-top plastic tubes containing Dulbecco's PBS solution and transported to the laboratory at 20-25°C (room temperature, RT) within 2 h after being removed. Immature oocytes were collected from ovaries scraping follicles into Petri dish containing Hepes-TCM-199 (1mg mL-1 PVA, 6 IU mL1 heparin, 100 IU mL -1 penicillin and 100 IU mL -1 streptomycin). Oocytes that were degenerated and/or with expanded cumulus cells were removed. Those with dark homogeneous cytoplasm and surrounded by packed cumulus cells were selected and randomly placed in four-well plates containing 500 µL of TCM 199 with 10%FCS and 24,2 mg mL -1 sodium pyruvate, 0,1 mM cysteamyn, 0,05 IU mL -1 FSH and 0,05 mL -1 LH, 100 IU mL -1 penicillin and 100 IU mL -1 streptomycin) in 4-well dishes (Nunc, Nunclon, Denmark) under mineral oil.

In vitro fertilization

After 24 h, cumulus cells were removed by gentle pipetting in H-TCM199 with hyaluronydase. Mature oocytes were selected and washed three times with SOF with 2% of oestrous sheep serum. Then, the oocytes were transferred into 4-well dishes containing 450 µL of synthetic oviduct fluid (SOF). Frozen electroejaculated semen from adult fallow deer was used for IVF. Spermatozoa in 0.25 ml pellets were thawed at 37 °C for 30 s, transferred to a glass tube with 1 ml SOF medium, and incubated for 40 min at 38.5 °C under 5% CO_2 in air. Only the upper 0,6 ml was collected, evaluated and used for fertilization.

Fallow deer semen and sheep selected oocytes were coincubated in four well with 0,45 ml of IVF-SOF till 18 h at 38.5 °C in 5% CO^2, 5% O^2 and 90% N^2 in humidified atmosphere.

In vitro culture

Presumed zygotes were washed three times in SOFaaBSA (SOFessential and non-essential amino acids) at oviductal concentration (Walker *et al*. 1996), 0.o4% BSA under mineral oil, in maximum humidified atmosphere with 5% CO2, 5% O2 and 90% N2 at 38.58C. and cultured in the same medium in 5% CO^2, 5% O^2 and 90% N^2 humidified atmosphere for 9 days. At 24h and 48h cleavage rates was evaluated

Sperm oocytes binding assay

Sperm-oocyte complexes were then incubated for 18 hours in 5% CO2 in air at 38°C and 100% humidity. After incubation, no cleaved sperm-oocyte complexes were pipetted three times with a 0.7 mm pipette in 100 µL droplets of phosphate-buffered saline (PBS) with 0.5% BSA to remove loosely attached spermatozoa. Before evaluation the complexes were stained for 15 minutes in a solution of 30 µL propidium iodide in 500 µL PBS with 0.5% BSA, at 38°C. oocytes were

transferred into 4-well plastic dishes (Nunc, Roskilde, Denmark) containing Hoechst 33342 and coincubated (20 min; room temperature (RT)) while protected from light. Oocytes were transferred onto a clean glass slide and compressed using cover slips. Each slide was examined immediately using fluorescent microscopy (X 40) to determine the number of sperm attached to penetrating the zona pellucida of each oocyte, and to assess the nuclear maturational status. The sperm-oocyte complexes were placed on a glass slide and slightly compressed by a cover slip, with a dot of vaseline in each corner. The spermatozoa bound to the ZP were counted with epifluorescence ultraviolet (UV) illumination. Penetrated oocytes were defined as those having sperms heads in the perivitelline space or in oocyte cytoplasm.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 17.0 (SPSS Inc, Chicago, III).

Results

Oocyte

There were 45-65 oocytes evaluated per male, with a total of 235 used in the study.

Semen

Characteristics of sperm samples after thawing are shown in Table

Sperm characteristics	(Medium value)±SD
Motile	49,67 ± 1,63
Progressive	12,00 ± 0,72
VAP	40,92 ± 2,35
VSL	29,22 ± 0,38
VCL	77,44 ± 5,88
ALH	8,08 ± 0,13
BCF	14,10 ± 0,57
STR	70,56 ± 3,03
LIN	41,22 ± 2,99
Elong	42,44 ± 2,18
Area	33,43 ± 2,86
Rapid	29,56 ± 3,51
Medium	20,00 ± 3,40
Slow	43,44 ± 4,42
Static	7,00 ± 2,62

Penetration, fertilization and cleavage rate

	Medium Value(%)±SEM
Penetration Rate	35 ± 6,82
Fertilization	31 ± 5,37
Cleavage	14 ± 11,85

Cleavage rate range from 13,7 % to 28,9% . The mean cleavage rate for all males was 14% ±11,85. Penetration and fertilization rates are shown in the table. Differences in fertility rates among males were not significant($p>0,05$).

Correlation among penetration, fertilization, cleavage

We found high correlation between binding of fallow deer spermatozoa to sheep oocytes and homologous IVF ($r= 0,998$ $p=0,002$). Total numbers of sperm cells binding to the zonae Moreover binding could be correlated to penetration rates ($r=0,979$ $p=0,021$). No correlation was found between cleavage in homologous and heterologous IVF.

Heterologous fertilization rates is negative correlated with static spermatozoa ($r=-0,991$; $p=0,009$).

Spermatozoa bound to the ZP of GV and MII oocytes. Binding was significantly ($p<0,0001$) different between GV and MII oocytes.

Discussion

In the study we correlated the relationship between sperm parameters, heterologous IVF and homologous IVF. We found that binding assay in a interfamiliae heterologous IVF could be considered a useful tool to predict fertility in IVF.

Cross-species fertilization of oocytes from domestic farm species has proven successful using cryopreserved sperm from deer (Comizzoli et al, 2001b), and nondomestic Bovidae (McHugh and Rutledge, 1998; Roth et al, 1999). Recently, sperm function was evaluated in an endangered African antelope, the scimitar-horned oryx (Oryx dammath), by means of a heterologous IVF system with zona-

intact domestic cow oocytes (Roth et al, 1998; 1999). Human sperm function is assessed by the sperm penetration assay (SPA) with zona-free hamster oocytes (Yanagimachi, 1984), and also has been used in several domestic species (Davis et al, 1987; Berger and Parker, 1989; Hammitt et al, 1989; Choudhry et al, 1995; Berger et al, 1996) and wild species (Lambert et al, 1991).

As previously showed, regarding heterologous IVF of zona-free bovine oocytes with deer spermatozoa, no relationship was found between standard semen characteristics and in vitro penetration (Comizzoli et al, 2001b). Generally few relationships were found between standard sperm characteristics and in vitro penetration ability (Martinez et al, 1996), and when they were significant, the regression coefficient was low (Hammitt et al, 1989). Few single sperm parameters appear to correlate significantly with in vitro penetration, especially when the quality of the samples is within acceptable ranges of normality. In addition, it has been proposed that the lack of correlation between conventional sperm parameters and the sperm penetration suggest that these assays measure different aspects of the viability and fertilizing capacity of spermatozoa (Jeyendran et al, 1984).

Binding assay has been proved to be an useful tool to evaluate activity of semen: During the active period of the seasonal viscacha reproductive cycle, sperm showed a high binding capacity for zona-free hamster oocytes. During the period of gonadal regression, the population of viscacha spermatozoa demonstrated a reduced binding to zona pellucida and denuded oocytes, although they did not show any significant difference in the parameters of morphology, motility and vitality between the periods studied (Merlo A. et al., 2000). In previous study on red deer (Soler and Garde, 2003) relationship between motility index and cleaved oocytes were found.

Moreover, it has been proposed that the lack of correlation among the conventional sperm parameters and assays of gamete binding suggest that these assays measure different aspects of the viability and fertilizing capacity of the spermatozoa (Jeyendran et al., 1984). Gamete recognition or primary binding between cells of the same species needs glycoproteins associated with the oocyte zona pellucida that recognize and establish a chemical link with complementary protein receptors of the spermatozoon(Bedford, 1991;Yanagimachi, 1994). Pre fertilization binding of spermatozoa to the zona pellucida is a receptor-mediated

event that involves cell surface interactions of sperm cell carbohydrate-binding proteins and glycoconjugates of the zona (reviewed by Wassarman, 1988; Saling, 1989). This receptor-mediated process results in acrosome membrane exocytosis and is regulated by agonists in the zona itself (Florman and Storey, 1982). Heterologous inter-gamete binding occurs only rarely. In vitro analysis of species specificity of fertilization indicate that sperm capacitation and the physiological affinity between the sperm of one species and the vitellus of another may be important limiting factors, but the zona pellucida appears to be a major block to interspecific fertilization (Hanada and Chang, 1976; Juneja et al., 1998).

However, other recent studies on thawed samples have obtained same results above all in no domestic species such as oryx (Roth TL, 1999). Motility of spermatozoa is an insufficient predictor of in vitro fertility in many species and is an inadequate measure of its functional competence (Amann RP, 1989; Roth TL 1998). Recently heterologous IVF have been used to assess the fertility of thawed ram spermatozoa comparing results of heterologous in vitro IVF with homologous *in vivo* AI. (O. Garcıa-Alvarez, 2010).

However, interspecies mating is successful only between a few closely related species due to natural barriers or isolating mechanisms (McGovern, 1976). Among the domestic bovids, there are several examples of hybrids resulting from interspecies breeding. European domestic cattle (Bos taurus) successfully interbreed with zebu cattle (Bos indicus), and American bison (Bison bison) has been hybridized with European bison (Bison bonasus) and domestic cattle (Basrur, 1986). The domestic goat (Capra hircus) is able to hybridise with Barbary sheep (Ammotragous cerviae) albeit at a low frequency (Anderson, 1988). Goats bred to sheep conceive but the hybrid pregnancies are generally lost by week 6 of gestation (Kelk et al., 1997a), although a few live-born goat–sheep hybrids have been documented (Bunch et al., 1976; Basrur, 1986; Anderson, 1988). cattle and sheep gametes undergo fertilisation and the resulting hybrid embryos develop to the 8-cell stage (Kelk, 1997; Slavik et al., 1997).

In conclusion, heterologous IVF can be useful to assess the functionality of sperm in endangered species, especially when it is not feasible or practical to collect large numbers of oocytes. However not only cleavage rate but also binding sperm-oocyte is a good indicator of in vitro fertility.

Conclusion

Reproductive biotechnologies have great potential as a tool for the conservation of endangered mammal species. We tried to apply some assisted reproductive techniques (ART) in the sardinian regional context, widely considered for its peculiar biodiversity. Our research had been driven in particular at cervids, Fallow deer (*Dama dama*, Linneus 1758) and Sardinian red deer (*Cervus elaphus corsicanus*, Exrleben 1777). This subspecies is strictly protected and is included as a Near Threatened on the IUCN Red List. First of all, we set up a genetic resource bank. Our first aim has been the preservation of gametes and somatic cells not only for storage but also for the application on somatic cell nuclear transfer. Sheep oocytes have been used as cytoplast, while fallow deer and Sardinian red deer as caryoplast. In vitro production of fallow deer embryos has been obtained in cloning but also thank to in vitro fertilization(IVF) of homologous oocytes. Semen had been previously tested for fertility in heterologous IVF with sheep oocytes. Our results showed that ART applications preserves the current genetic diversity, gives future reproductive opportunities and could allow more rapid expansion of a population. In conclusion, the study of

reproduction is fundamental for conserving species, populations and, indirectly, the vitality of entire ecosystems. However, at the moment, a successful management of wildlife depends also on intensive genetic and reproductive management.

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References

Apollonio M, Andersen R, Putman R eds European Ungulates and Their Management in the 21 st Century. *Cambridge University Press*, Cambridge, UK, 2010.

Ciucci P, Catullo G, Boitani L .Do counts of roaring stags index red deer (*Cervus elaphus*) population size? *Wildl Res*, 2009

David J. The International Year of Biodiversity–From Talk to Action. *Conservation Biology* 2009;24 (1): 338–340;

Ente Foreste della Sardegna (2010) Censimento 2010 del Cervo sardo. Regione Autonoma della Sardegna. Available at http://www.sardegnaforeste.it

Gómez MC, Serrano MA, Pope CE, Jenkins JA, Biancardi MN, López M, Dumas C, Galiguis J, Dresser BL. Derivation of cat embryonic stem-like cells from in vitro-produced blastocysts on homologous and heterologous feeder cells. *Theriogenology.* 2010 Sep 1;74(4):498-515.

Hanks J. Conservation strategies for Africa's large mammals. *Reprod Fertil Dev*. 2001;13(7-8):459-68.

Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. *Rev Reprod.* 1999 Sep;4(3):143-50.

Jewgenow K, Paris MC. Preservation of female germ cells from ovaries of cat species. *Theriogenology.* 2006 Jul 1;66(1):93-100.

Leon-Quinto T, Simon MA, Cadenas R, Jones J, Martinez-Hernandez FJ, Moreno JM, Vargas A, Martinez F, Soria B Developing biological resource banks as a supporting tool for wildlife reproduction and conservation The Iberian lynx bankas a model for other endangered species. *Anim Reprod Sci.* 2009 ;112(3-4):347-61.

Lloyd J "Biodiversity". In: Encyclopedia of Earth. Eds. Cutler J. Cleveland (Washington, D.C.: *Environmental Information Coalition*, National Council for Science and the Environment). December 28, 2009;

Loi P, Fulka J Jr, Hildebrand T, Ptak G. Genome of non-living cells: trash or recycle? *Reproduction.* 2011 Oct;142(4):497-503.

Loskutoff N.M. Role of embryo technique in genetic management and conservation of wildlife. In "Reproductive science and Integrated conservation"(Eds Holt W.V., Pickard A.R., Roger J.C., Wildt D.E.)pp 183-194 *Cambridge University* –Cambridge UK. 2003

Lovari, S., Herrero. J., Conroy, J., Maran, T., Giannatos, G., Stubbe, M., Aulagnier, S., Jdeidi, T., Masseti, M. Nader, I., de Smet, K. & Cuzin, F. 2008. *Cervus elaphus*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.1

Margules CR, Pressey RL Systematic conservation planning. *Nature*. 2000 ;11;405(6783):243-53.

Meintjes M, Bellow MS, Broussard JR, Paul JB, Godke RA. Transvaginal aspiration of oocytes from hormone-treated pregnant beef cattle for in vitro fertilization. *J Anim Sci.* 1995 Apr;73(4):967-74.

Pope CE. Embryo technology in conservation efforts for endangered felids. *Theriogenology*. 2000 Jan 1;53(1):163-74

Puddu G.,Maiorano L., Falcucci A., Corsi F., Boitani L. Spatial-explicit assessment of current and future conservation options for the endangered Corsican Red Deer (*Cervus elaphus corsicanus*) in Sardinia. *Biodivers Conserv* 2009

Pukazhenthi BS, Wildt DE. Which reproductive technologies are most relevant to studying, managing and conserving wildlife? *Reprod Fertil Dev*. 2004;16(1-2):33-46.

Sutmoller P, Wrathall AE. The risks of disease transmission by embryo transfer in cattle. *Rev Sci Tech.* 1997 Apr;16(1):226-39.

Wildt DE, Ellis S, Howard JG. Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. *J Reprod Fertil Suppl*. 2001;57:295-307.

Zachos F.E., Hartl G.B. Phylogeography, population genetics and conservation of the European red deer *Cervus elaphus*. *Mammal Review.* Volume 41, Issue 2, pages 138–150, April 2011

Cloning

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek

CA, Meade HM, Godke RA, Gavin WG, Overström EW, Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol*. 1999 May;17(5):456-61.

Bowles EJ, Lee JH, Alberio R, Lloyd RE, Stekel D, et al. Contrasting effects of in vitro fertilization and nuclear transfer on the expression of mtDNA replication factors. *Genetics* 2007; 176: 1511–1526

Briggs R, King TJ Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc Natl Acad Sci U S A*. 1952; 38(5):455-63.

Bui LC, Vignon X, Campion E, Laloy E, Lavergne Y, Ty LV, et al. Use of interspecies nuclear transfer to study the early embryonic development and nuclear activities of the endangered species Pseudoryxnghetinhensis (saola). *Theriogenology* 2002;57:427

Campbell KH, McWhir J, Ritchie WA, Wilmut I.Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 1996:380(6569):64-6.

Chang KH, Lim JM, Kang SK, Lee BC, Moon SY, et al. Blastocyst formation, karyotype, and mitochondrial DNA of interspecies embryos derived from nuclear transfer of human cord fibroblasts into enucleated bovine oocytes. *Fertil Steril* 2003; 80: 1380–1387

Chen D, Wen D, Zhang Y, Sun Q, Han Z, Liu Z, Shi P, Li J, Xiangyu J, Lian L, Kou Z, Wu Y, Chen Y, Wang P, Zhang H. Interspecies implantation and mitochondria fate of panda-rabbit cloned embryos. *Biol. Reprod*. 2002; 67: 637-642

Chesné P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP.Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat Biotechnol* 2002 Apr;20(4):366-9

Chung Y, Bishop CE, Treff NR, Walker SJ, Sandler VM, et al. Reprogramming of human somatic cells using human and animal oocytes. *Cloning Stem Cells* 2009; 11: 213–223.

Cibelli JB, Kiessling AA, Cunniff K, Richards C, Lanza RP, West MD. Somatic cell nuclear transfer in humans: Pronuclear and early embryonic development. *J Regenerative Med* 2001;2:25–31

Dey R, Barrientos A, Mraes CT. Functional constraints of nuclear-mitochondrial DNA interaction in xenomitochondrial rodent cell lines. *J Biol Chem* 2000;275:31520–7.

Dominko, T., Mitalipova, M., Haley, B., Beyhan, Z., Memili,E., McKusick, B. & First, N.L. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. *Biol. Reprod*. 1999; 60, 1496–1502

Edwards JL, Dorado CM, Wilson TJ, Schrick FN.Development of cloned embryos reconstructed with serum fed or serum starved adult granulosa cells. *Theriogenology* 2001; 55:265

Egli D, Rosains J, Birkhoff G, Eggan K. Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature* 2007;447:679–685

Folch, J., Cocero, M.J., Chesne, P., Alabart, J.L., Dominguez, V.,Cognie, Y., Roche, A., Fernandez-Arias,A., Marti, J.I., Sanchez,P.,Echegoyen, E., Beckers,J.F.,Bonastre, A.S., Vignon, X. First birth of an animal from an extinct subspecies (Capra pyrenaica pyrenaica) by cloning. *Theriogenology* 2009;71: 1026-1034.

Galli, C., Lagutina, I., Crotti, G., Collenoni, S., Turini, P., Ponderato,N., Duchi, R., Lazzari, G.,. Pregnancy: a cloned horse born to its dam twin. *Nature* 2003;424: 635

Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg R, Respess D, Venable A, Stice S. Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. *Biol Reprod* 2002; 66:895–900

Go´mez MC, Jenkins JA, Giraldo A, Harris RF, King A, Dresser BL, Pope CA. Nuclear transfer of synchronized African wild cat somatic cells into enucleated domestic cat oocytes. *Biol Reprod*. 2003;69:1032–41.

Damiani P, Wirtu G, Miller F, Cole A, Pope C, Godke RA, et al. Development of giant eland (Taurotragus oryx) and bovine (Bos taurus) oocytes. *Theriogenology* 2003;59:390

Jiang Y, Liu SZ, Zhang YL, Jiang MX, Sun QY, et al. The fate of mitochondria in Ibex-hirus reconstructed early embryos. *Acta Biochim Biophys Sin* (Shanghai) 2004; 36: 371–374

Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J Reprod Fertil.* 2000; 120:231–237

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science*. 1998; 11;282(5396):2095-8.

Kikyo N, Wolffe AP: Reprogramming nuclei: insights from cloning, nuclear transfer and heterokaryons. *J Cell Sci.* 2000,113(Pt 1):11-20

Kim M, Jang G, Oh H, Yuda F, Kim H, Hwang W, Hossein M, Kim J,Shin N, Kang S. Endangered wolves cloned from adult somatic cells. *Cloning Stem Cells* 2007;9: 130-137.

Kishi M, Itagaki Y, Takakura R, Imamura M, Sudo T,Yoshinari M, Tanimoto M, Yasue H, Kashima N.Nuclear transfer in cattle using colostrum-derived mammary gland epithelial cells and ear–derived fibroblast cells. *Theriogenology* 2000; 54:675–684

Kucharski R, Maleszka J, Foret S, Maleszka R. Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 2008;319:1827–1830

Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, et al. Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. *Cloning* 2000;2:79–90.

Lee,B.C., Kim, M.K., Jang, G., Oh, H.J., Yuda, F., Kim,H.J., Hossein, M.S.,Kim,J.J., Kang, S.K., Schatten, G., Hwang, W.S.. Dogs cloned from adult somatic cells. *Nature* 2005; 436: 641.

Li GP, Siedel GE, Squires EL. Interspecies cloning using fresh, stored and dead equine and bovine somatic cells as donor nuclei and bovine cytoplasts. *Theriogenology* 2002;57:432

Li Z., Sun X., Chen J., Liu X., Wisely S.M., Zhou Q., Renard J.P., Leno G.H., Engelhardt J.F.. Cloned ferrets produced by somatic cell nuclear transfer. *Dev Biol* 2006;293: 439–448.

Loi P, Ptak G, Barboni B, Fulka J, Cappai P, Clinton M. Genetic rescue of an endangered mammal by crossspecies nuclear transfer using post-mortem somatic cells. *Nature Biotech* 2001;19:962–4.

Kim M, Jang G, Oh H, Yuda F, Kim H, Hwang W, Hossein M, Kim J, Shin N, Kang S. Endangered wolves cloned from adult somatic cells. *Cloning Stem Cells*, 2007;9: 130-137.

Østrup O, Strejcek F, Petrovicova I, Lucas-Hahn A, Morovic M, Lemme E, Petersen B, Laurincikova N, Niemann H, Laurincik J, Hyttel P..Role of ooplasm in nuclear and nucleolar remodeling of intergeneric somatic cell nuclear transfer embryos during the first cell cycle. *Cell Reprogram*. 2011 Apr;13(2):145-55

Meirelles FV, Bordignon V, Watanabe Y, Watanabe M, Dayan A, et al. Complete replacement of the mitochondrial genotype in a Bos indicus calf reconstructed by nuclear transfer to a Bos taurus oocyte. *Genetics* 2001;158: 351–356.

Miyashita N, Shiga K, Yonai M, Kaneyama K, Kobayashi S, Kojima T, Goto Y, Kishi M, Aso H, Suzuki T, Sakaguchi M, Nagai T. Remarkable differences in telomere lengths among cloned cattle derived from different cell types. *Biol Reprod*. 2002 Jun;66(6):1649-55.

Piedrahita JA, Wells DN, Miller AL, Oliver JE, Berg MC, Peterson AJ, Tervit HR. Effects of follicular size of cytoplast donor on the efficiency of cloning in cattle. *Mol Reprod Dev* 2002; 61:317–326

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH.Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 2000; 7;407(6800):86-90.

Sansinena MJ, Reggio BC, Denniston RS, Godke RA. Nuclear transfer embryos from different equine cell lines as donor karyoplasts using the bovine oocyte as recipient cytoplast. *Theriogenology* 2002;58:775–7.

Shi,D., Lu,F., Wei,Y., Cui,K., Yang,S., Wei, J., Liu,Q.. Buffalos (Bubalus bubalis) cloned by nuclear transfer of somatic cells. *Biol Reprod* 2007;77: 285-291

Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M.A cat cloned by nuclear transplantation. *Nature* 2002 Feb 21;415(6874):859.

Simonsson S, Gurdon J. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. *Nature Cell Biol* 2004;6:984–990

Sowers, A.E.. Evidence that electrofusion yield is controlled by biologically relevant membrane factors. *Biochim. Biophys. Acta* 1989;985, 334–8.

Spemann E. Embryonic Development and Induction. *Yale University Press*, New Haven. 1938

St John JC, Lloyd RE, Bowles EJ, Thomas EC, El Shourbagy S. The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. *Reproduction* 2004;127:631–641

St John JC, Moffatt O, D'Souza N. Aberrant heteroplasmic transmission of mtDNA in cloned pigs arising from double nuclear transfer. *Mol Reprod Dev* 2005;72: 450–460.

Surani A. Reprogramming of genome function through epigenetic inheritance. *Nature* 2001; 414:122–128.

Takeda K, Akagi S, Kaneyama K, Kojima T, Takahashi S, et al.Proliferation of donor mitochondrial DNA in nuclear transfer calves (Bos taurus) derived from cumulus cells. *Mol Reprod Dev* 2003; 64: 429–437.

Thongphakdee A, Kobayashi S, Imai K, Inaba Y, Tasai M,Tagami T, et al. Interspecies nuclear transfer embryos reconstructed from cat somatic cells and bovine ooplasm. *J Reprod Dev* 2008;54:142–7.

Ty LV, Hanh NV, Uoc NT, Duc NH, Thanh NT, Bui LC, et al. Preliminary results of cell cryobanking and embryo production of black bear (Ursus thibetanus) by interspecies somatic cell nuclear transfer. *Theriogenology* 2003;59:290

Tong D.Z, Wu S.Q, Ye Y.F, Yang S.Y, Du M, Lu D.Y *Nuclear transfer in fishes Sci.* Bull1963; **7** 60–61.

Do JT, Lee JW, Lee BY, Kim SB, Ryoo ZY, et al. Fate of donor mitochondrial DNA in cloned bovine embryos produced by microinjection of cumulus cells. *Biol Reprod* 2002; 67: 555–560.

Takeda K, Tasai M, Iwamoto M, Akita T, Tagami T, et al. Transmission of mitochondrial DNA in pigs and progeny derived from nuclear transfer of Meishan pig fibroblast cells. *Mol Reprod Dev* 2006;73: 306–312.

Tani T, Kato Y, Tsunoda Y. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biol Reprod* 2001; 64:324–330

Tong D.Z, Ye Y.H, Lu D.Y, Tong S.M, Du M Transplantation of nuclei between two subfamilies of teleosts (Goldfish-domesticated carassius and Chinese bitterling *Rhodeus sinensis*) *Acta Zool. Sin* 1973;19 201–207;

Vrana PB, Guan XJ, Ingram RS, Tilghman SM. Genomic imprinting is disrupted in interspecific Peromyscus hybrids. *Nat Genet* 1998;20:362–5.

Yin X, Lee Y, Lee H, Kim N, Kim L, Shin H, Kong I . In vitro production and initiation of pregnancies in inter-genus nuclear transfer embryos derived from leopard cat (Prionailurus bengalensis)nuclei fused with domestic cat (Felis silverstris catus) enucleated oocytes. *Theriogenology*, 2006;66: 275-282.

Yoon JT, Choi EJ, Han KY, Shim H, Roh S. In vitro development of embryos produced by nuclear transfer of porcine somatic cell nuclei into bovine oocytes using three different culture systems. *Theriogenology* 2001;55:298

Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R.Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; 23;394(6691):369-74.

Wang ZG, Wang W, Yu SD, Xu ZR.. Effects of different activation protocols on preimplantation development, apoptosis and ploidy of bovine parthenogenetic embryos. *Anim Reprod Sci*. 2008 ;105(3-4):292-301.

Wani,N.A., Wernery,U., Hassan,F.A., Wernery,R., Skidmore,J.A..Production of the first cloned camel by somatic cell nuclear transfer. *Biol Reprod* 2010;82: 373-379.

Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod* 1999; 60:996–1005.34.

Wells DN, Misica PM, Tervit HR, Vivanco WH. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod Fertil Dev* 1998; 10:369–378.

White KL, Bunch TD, Mitalipov S, Reed WA. Establishment of pregnancy after the transfer

of nuclear transfer embryos produced from the fusion of argali (Ovis ammon) nuclei into domestic sheep (Ovis aries) enucleated oocytes. *Cloning* 1999;1:47–54.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH Viable offspring derived from fetal and adult mammalian cells.Nature. 1997 Feb 27;385(6619):810-3. Erratum in: *Nature* 1997; 13;386(6621):200.

Woods,G.L., White,K.L., Vanderwall,D.K., Li,G.P., Aston,K.I.,Bunch,T.D., Meerdo,L.N., Pate,B.J.. A mule cloned from fetal cells by nuclear transfer. *Science* 2003;301: 1063

Zakhartchenko V, Alberio R, Stojkovic M, Prelle K,Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R,Duchler M, Steinborn R, Meuller M, Brem G, Wolf E.Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. *Mol Reprod Dev* 1999; 54:264–272

Zhao ZJ, Ouyang YC, Nan CL, Lei ZL, Song XF, Sun QY, Chen DY. Rabbit oocyte cytoplasm supports development of nuclear transfer embryos derived from the somatic cells of the camel and Tibetan antelope. *J Reprod Dev* 2006; 52: 449–459

Zhou,Q., Renard,J.P., Le,Friex,G., Brochard,V., Beaujean, N.,Cherifi,Y., Fraichard,A., Cozzi, J. Generation of fertile cloned rats by regulating oocyte activation. *Science* 2003; 302: 1179

Somatic cell

Allsopp RC: Models of initiation of replicative senescense by loss of telomeric DNA. *Exp Gerontol* 1996, 31:235-243

Balin AK, Fisher AJ, Anzelone M, Leong I, Allen RG: Effects of establishing cell cultures and cell culture conditions on the proliferative lifespan of human fibroblasts isolated from different tissues and donors of different ages. *Exp Cell Res* 2002, 274:275-287.

Bhojwani, S., Vajta, G., Callesen, H., Roschlau, K., Kuwer, A., Becker, F., Alm, H., Torner, H., Kanitz, W. & Poehland,R.. Developmental competence of HMCTM derived bovine cloned embryos obtained from somatic cell nuclear transfer of adult fibroblasts and granulosa cells. *J. Reprod. Dev*. 2005; 51, 465–75.

Burgoyne, P.S., Holland, K., and Stephens, R. . Incidence of numerical chromosome anomalies in human pregnancy estimation from induced and spontaneous abortion data. *Hum. Reprod*. 1991;6, 555–565.

Byrne, J.L.B., and Ward, K. . Genetic factors in recurrent abortion. *Clin. Obstet. Gynecol*. 1994;37, 693–704

Bureau W, Bordignon V, Leveillee C, Smith LA, King A. 2003. Sexchromosomal complement of donor cells and reconstructed embryos derived from them. *Theriogenology* 59:239

Campisi J, Dimri GP, Nehlin JO, Testori A, Yoshimoto K: Coming of age in culture. *Exp Gerontol* 1996, 31:7-12.

Chacon L, Gomez Martha C, Jenkins J. A., Leibo S.P.3,4,Wirtu G3,Dresser B.D. and C.Pope C.E. Production of bovine cloned embryos with donor cells frozen at a slow cooling rate in a conventional freezer (−20 ◦C) *Zygote* 17 (November), pp. 341–351.

Chu EH.. Chromosomal stabilization of cell strains. *Natl Cancer Inst Monogr* 1962;7:55–71.

Denning C, Dickinson P, Burl S, Wylie D, Fletcher J, Clark AJ..Gene targeting in primary fetal fibroblasts from sheep and pig. *Cloning Stem Cells* 2001;3:221–23

Dessauer, H. C., Cole, C. J. & Hafner, M. S.:Collection and storage of tissues. In Molecular systematics (2nd edn). 29–47. Hillis, D. M., Moritz, C. M.& Mable, B. K. (Eds). *Sunderland,* MA: Sinauer Associates;1996.

Du, F., Sung, L.Y., Tian, X.C. & Yang, X.. Differential cytoplast requirement for embryonic and somatic cell nuclear transfer in cattle. *Mol. Reprod. Dev.* 2002;**63**, 183–91.

Ford DK, Wakonig R, Yerganian G.. Further observations on the chromosomes of Chinese hamster cells in tissue culture. *J Natl Cancer Inst* 1959;22:765–799.

Freshney RI. Cell line provenance. *Cytotechnology*. 2002 Jul;39(2):55-67.

Giraldo, A.M., Lynn, J.W., Godke, R.A. & Bondioli, K.R.. Proliferative characteristics and chromosomal stability of bovine donor cells for nuclear transfer. *Mol.Reprod. Dev*2006; **73**, 1230–8.

Gomez M.C., Pope,C.E., Lopez, M.,Dumas, C.,Giraldo,A. &Dresser, B.L.. Chromosomal aneuploidy in African Wildcat somatic cells and cloned embryos. *Cloning Stem Cells* 2006;8, 69–78.

Green, A.L., Wells, D.N. & Oback, B. . Cattle cloned from increasingly differentiated muscle cells. *Biol. Reprod*. 2007;77, 395–406.

Hayflick L, Moorhead PS: The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961, 25:585-621.

Hoshino Y.,Hayashi N., Taniguchi S., Kobayashi N., Sakai K.,Otani T., Iritani A., Saeki K. Resurrection of a Bull by Cloning from Organs Frozen without Cryoprotectant in a -80°C Freezer for a Decade. *Plos One* 2009;4 (1)

Hsu TC, Kellogg DS. Mammalian chromosomes in vitro. XII. Experimental evolution of cell populations. *J Natl Cancer Inst* 1960; 24: 1067–1093.

Hsu TC, Kellogg DS.. Primary cultivation and continuous propagation in vitro of tissues from small biopsy specimens. *J Natl Cancer Inst* 1960b; 25:221–235.

Kato, Y., Tani, T. & Tsunoda, Y.. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J. Reprod. Fertil* 2000*.;* **120**, 231–7.

Kragh, P.M., Du, Y., Corydon, T.J., Purup, S., Bolund, L. & Vajta, G.. Efficient *in vitro* production of porcine blastocysts by handmade cloning with a combined electrical and chemical activation. *Theriogenology* 2005;**64**, 1536–45.

Kragh, P.M., Vajta, G., Corydon, T.J., Purup, S., Bolund, L.& Callesen, H. . Production of transgenic porcine blastocysts by hand-made cloning. *Reprod. Fertil. Dev.* 2004;**16**,315–8.

Kubota, C., Yamakuchi, H., Todoroki, J., et al. . Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc. Natl. Acad. Sci.* USA 2000;97, 990–995

Levan A, Hauschka TS.. Endomitotic reduplication mechanisms in ascites tumors of the mouse. *J Natl Cancer Inst* 1953;14:1 – 43.

Loi P, Ptak G, Barboni B, Fulka J Jr, Cappai P, et al. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat Biotechnol* 2001;19: 962–964.

Loi P, Clinton M, Barboni B, Fulka J Jr, Cappai P, et al. Nuclei of nonviable ovine somatic cells develop into lambs after nuclear transplantation. *Biol Reprod* 2002;67: 126–132.

Loi P, K Matzukawa, G Ptak, Y Natan, J Fulka Jr, A Arav. Nuclear Transfer of Freeze-Dried Somatic Cells into Enucleated Sheep Oocytes. Reprod.Dom.Anim. 2008;43, (2), 417–422,

Muldrew, K., Acker, J.P., Elliot, J.A.W. & McGann, E. . The water to ice transition: implications for living cells. In *Life in the Frozen State* (eds B.J. Fuller, N. Lane & E.E. Benson) 2004;pp. 67–108. Boca Raton: CRS Press

Oback B, Wells D.. Donor cells for nuclear cloning: Many are called, but few are chosen. *Cloning Stem Cells* 2002;4:147–168.

Ota T, Suto S, Katayama H, Han ZB, Suzuki F, Maeda M, Tanino M, Terada Y, Tatsuka M. Increased mitotic phosphorylation of histone H3 attributable to AIM-1/Aurora-B overexpression contributes to chromosome number instability. *Cancer Res* 2002;62:5168–5177.

Pignolo RJ, Cristofalo VJ, Rotenberg MO: Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. *J Biol Chem* 1993, 268:8949-8957

Revel A, Elami A, Bor A, Yavin S, Natan Y, Arav A. Whole sheep ovary cryopreservation and transplantation. *Fertil Steril.* 2004 Dec;82(6):1714-5.

Smith, L. M. & Burgoyne, L. A. Species identity:conserved inverted LINE repeat clusters (ILRC) in the vertebrate genome as indicators of population boundaries. *Gene* 2001;271: 273–283.

Tani, T., Kato, Y. & Tsunoda, Y.. Developmental potential of cumulus cell-derived culture frozen in a quiescent state after nucleus transfer. *Theriogenology* 2000;**53**,1623–9.

Vink, C. J., Thomas, S. M., Paquin, P., Hayashi, C. Y. &Hedin, M. The effects of preservatives and temperatures on arachnid DNA. *Invertebrate Systematics* 2005;19: 99–104

Wells D.N., Misica, P.M. & Tervit, H.R. . Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod* 1999; **60**, 996–1005

Wildt DE: Genetic resource banks for conserving wildlife species: justification, examples and becoming organized on a global basis. *Anim Reprod Sci* 1992, 28:247-257.

Wilmut I., Schnieke,A.E., Mcwhir,J., Kind,A.J., Campbell,K.H. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;385: 810–813.

Zhu H, Tamot B, Quinton M, Walton J, Hacker RR, Li J: Influence of tissue origins and external microenvironment on porcine foetal fibroblast growth, proliferative life span and genome stability. *Cell Prolif* 2004, 37:255-266.

IVF

Apollonio M, Festa-Bianchet M, Mari F, Mattioli S, Sarno S. To lek or not to lek: mating strategies of male fallow deer. *Behavioural Ecology*, 1992;3, 25–31.

Armstrong, N., Chaplin, R.G., Chapman, D.I. & Smith, Observations on the reproduction of female wild and park Fallow deer (*Dama dama*) in southern England. *J. Zool, Lond*. 1969;158, 27-37

Asher GW. Oestrous cycle and breeding season of farmed fallow deer, *Dama dama*. *J Reprod Fertil.* 1985 Nov;75(2):521-9.

G.W. Asher, K.T. O'Neill, I.C. Scott, B.G. Mockett and A.J. Pearse , Genetic influences on reproduction of female red deer (*Cervus elaphus*). (2). Seasonal and genetic effects on the superovulatory response to exogenous FSH. *Anim. Reprod. Sci*. 2000, **59** pp. 61–70

Asher GW. Reproductive cycles of deer. *Anim Reprod Sci.* 2011 Apr;124(3-4):170-5. Epub 2010 Sep 29.

Baker, K. Reproduction biology of fallow deer (*Dama dama*) in the Blue Mountains of New Zealand. M.Sc. thesis, 1973 University of Otago

Bavister, B.D., 1975. Properties of the sperm motility-stimulating component derived from human serum. *J.Reprod. Fertil.* 43, 363–366

D.K. Berg, J.G. Thompson, P.A. Pugh, H.R. Tervit and G.W. Asher, Successful in-vitro culture of early cleavage stage embryos recovered from superovulated red deer (*Cervus elaphus*), *Theriogenology* 1995; **44**, pp. 247–254

Berg DK, Thompson JG, Asher GW. Development of in vitro embryo production systems for red deer (*Cervus elaphus*). Part 1. Effect of epithelial oviductal monolayers and heparin on in vitro sperm motility and penetration of in vitro matured oocytes. *Anim Reprod Sci.* 2002 Mar 15;70(1-2):65-76

Berg DK, Thompson JG, Asher GW. Development of in vitro embryo production systems for red deer (*Cervus elaphus*). Part 2. The timing of in vitro nuclear oocyte maturation. *Anim Reprod Sci.* 2002 Mar 15;70(1-2):77-84.

Berg DK, Pugh PA, Thompson JG, Asher GW Development of in vitro embryo production systems for red deer (*Cervus elaphus*). Part 3. In vitro fertilisation using sheep serum as a capacitating agent and the subsequent birth of calves. *Anim Reprod Sci.* 2002 Mar 15;70(1-2):85-98.

D.K. Berg and G.W. Asher, New developments reproductive technologies in deer. *Theriogenology* 2003; **59** pp. 189–205.

Berg, D.K.,Thompson, J.G.,Peterson, A.J.,Asher, G.W.,.Thetemporal relationship between oocyte maturation and early fertilization events in relation to the pre-ovulatory LH peak and pre-implantation embryo development in red deer(Cervus elaphus). *Anim.Reprod.Sci*. 2008; 105, 332–343.

Brown, D.V., Senger, P.L.,. Influence of homologous blood serum on motility and head-to-head agglutination in non motile ejaculated bovine spermatozoa. *Biol. Reprod*.1980; 23, 271–275

Chapman, D.I. & Chapman, N. Observations on the biology of fallow deer (*Dama dama*) in Epping Forest, Essex, England. *Biological Conversation* 1969;2,55-62

Chapman, D.I. & Chapman, N. Fallow Deer: their History, *Distribution and Biology. Terence Dalton Ltd.*1975

S.A. Chapman, D.L. Keller, M.E. Westhusin, M.L. Drew and D.C. Kraemer, In vitro production of axis deer (Axis axis) embryos, a preliminary study. *Theriogenology* 1999;51 **,** p. 280

Clutton-Brock TH, Green D, Hiraiwa Hasegawa M, Albon SD () Passing the buck: resource defense, lekking and mate choice in fallow deer. *Behavioural Ecology and Sociobiology*, 1988; 23, 281–296.

P. Comizzoli, P. Mermillod, Y. Cognie, N. Chai, X. Legendre and R. Mauget, Successful in vitro production of embryos in the red deer and the sikadeer. *Theriogenology* 2001; **55**, pp. 649–659

Cowan, I. McT. Life and times of the coast blacktailed deer. In The Deer of North America,. Ed. W. P. Taylor. *Stackpole Co*., Washington, DC. 1965; pp. 523-617

Egerszegi I, HazelegerW, Ra´tky J, Sarlo´s P, Kemp B, Bouwman E, et al. Superovulatory ovarian response in Mangalica goats is not influenced by feeding level. *Reprod Domest Anim* 2007;42:441–4.

García-Alvarez O, Maroto-Morales A, Berlinguer F, Fernández-Santos MR, Esteso MC, Mermillod P, Ortiz JA, Ramon M, Pérez-Guzmán MD, Garde JJ, Soler AJ.Effect of storage temperature during transport of ovaries on in vitro embryo production in Iberian red deer (*Cervus elaphus hispanicus*). *Theriogenology* 2011 Jan 1;75(1):65-72. Epub 2010 Oct 30.

Haltenorth, T. Beitrag zur kenntnis des Mesopotamischen Damhirsches—Cervus (Dama) mesopotamicus Brooke, 1875—und zur stammes- und verbreitungsgeschichte der Damhirsche Allengemein. *Saugetierk* Mitt.Bd VIII, Sonderheft, 1959.1—89.

Heidemann, o.. Status of Turkish fallow deer (*Cervus dama dama*). mt. Symp. Wildlife Fauna in Turkey and in the Balkan Countries. *CIC. Istambul*. 1987

Jabbour HN, Marshall VS, Argo CM, Hooton J, Loudon AS. Successful embryo transfer following artificial insemination of superovulated fallow deer (*Dama dama*). *Reprod Fertil Dev.* 1994;6(2):181-5.

Krogenaes A, Ropstad E, Thomassen R, Pedersen O, Forsberg M. In vitro maturation and fertilization of oocytes from Norwegian semi domestic reindeer (Rangifer tarandus). *Theriogenology* 1994; 41:371-7

Locatelli Y., Y. Cognié, J.C. Vallet, G. Baril, M. Verdier, N. Poulin, X. Legendre and Mermillod, Succesful use of oviduct epithelial cell coculture for in vitro production of viable red deer (*Cervus elaphus*) embryos, *Theriogenology* 2005; 64, pp. 1729–1739

Mandal, M., Banerjee, S., Majumder, G.C., 1989. Stimulation of forward motility of goat cauda epididymal spermatozoa by a serum glycoprotein factor. *Biol. Reprod*. 41, 983–989

McElligott AG, Hayden TJ .Lifetime mating success, sexual selection and life history of fallow bucks (*Dama dama*). *Behavioral Ecology and Sociobiology*, 2000; 48, 203–210.

Moor, R.M., Crosby, I.M.,. Temperature-induced abnormalities in sheep oocytes during maturation. *J. Reprod. Fertil*. 1985;75, 467–473

Moore NP, Kelly PF, Cahill JP, Hayden TJ. Mating strategies and mating success of fallow (*Dama dama*) bucks in a non-lekking population. *Behavioral Ecology and Sociobiology*, 1995;36, 91–100.

Morrow CJ, Asher GW, Berg DK, Tervit HR, Pugh PA, McMillan WH, Beaumont S, Hall DR, Bell AC. Embryo transfer in fallow deer (*Dama dama*): superovulation, embryo recovery and laparoscopic transfer of fresh and cryopreserved embryos. *Theriogenology* 1994 Sep;42(4):579-90.

Nowak, r. M. And paradiso, J. L.. Mammals of the World. Vol II. *Johns Hopkins Univ*. Press, Baltimore and London. 1983

O'Connell JM. Reproduction in fallow does (*Dama dama*; Linnaeus, 1758) and fawn survival in Phoenix Park, Dublin. MSc Thesis, *The National University of Ireland*, Dublin. 1993

Pawshe, C.H., Appa Rao, K.B.C., Jain, S.K., Totey, S.M.,. Biochemical studies on goat oocytes: timing of nuclear progression, effect of protein inhibitor and pattern of polypeptide synthesis during in vitro maturation. *Theriogenology* 1994; 42, 307–320

Pemberton, j. M. And smith, R H.. Lack of biochemical polymorphism in British fallow deer. *Heredity* 1985; 55, 199-207.

Ptak G., M. Clinton, B. Barboni, M. Muzzeddu, P. Cappai and M. Tischner et al., Preservation of the wild European mouflon: the first example of genetic management using a complete program of reproductive biotechnologies, *Biol Reprod.* 2002;66, pp. 796–801

Sirard, M.A., Florman, H.M., Leibfried-Rutledge, M.L., Barnes, F.L., Sims, M.L., First, N.L.,. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol. Reprod* 1989; 40, 1257–1263

Siriaroonrat B., P. Comizzoli, N. Songsasen, S.L. Monfort, D.E. Wildt, B.S. Pukazhenthi. Oocyte quality and estradiol supplementation affect in vitro maturation success in the white-tailed deer (Odocoileus virginianus). *Theriogenology* 2010;73 112–119

Whitehead, G.K.The Whitehead Encyclopaedia of Deer. *Swan Hill Press*, Shrewsbury, UK. 1993

Heterologous IVF

Amann, RP. Can the fertility potential of a seminal sample be predicted accurately.

J Androl 1989;10: 89-98

Bedford JM. The coevolution of gametes. In: A Comparative Overview of Mammalian Fertilization. *B.S. Dunbar, M.G. O´Rand Eds*. Plenum Press, New York, 1991;pp. 3-50

Berger T, Parker K. Modification of the zona-free hamster ova bioassay of boar sperm fertility and correlation with *in vivo* fertility. *Gam Res* 1989;22:385–97

Bousquet D, Brackett BG. Penetration of zona-free hamster ova as a test to assess fertilizing ability of bull sperm after frozen storage. *Theriogenology* 1982;17:199–212.

Comizzoli P, Mauget R, Mermillod P,: Assessment on in vitro fertility of deer spermatozoa by heterologous IVF with zona-free bovine oocytes. *Theriogenology* 2001;56, 261–274

Franken DR. New aspects of sperm–zona pellucida binding. *Andrologia* 1998;30:263–8

Garcıa-Alvarez O.,Maroto-Morales A.,Martınez-Pastor F., Fernandez-Santos M.R., Esteso M.C., Perez-Guzman M.C., Soler A.J. Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. *Theriogenology* 2009;71 643–650

Kouba AJ, Atkinson MW, Gandolf AR, Roth TL,:Species-specific sperm-egg interaction affects the utility of a heterologous bovine in vitro fertilization system for evaluation antelope sperm. *Biol Reprod* 2001;65, 1246–1251

Jeyendran R, Van der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L. Development of an assay to asses the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil*. 1984;70: 219-228.

Juneja R, Agulnik S, Silver L. Sequence divergence within the sperm-specif polypeptide TCTE 1 is correlated with species-specific differences in sperm binding to zona-intact eggs. *J Androl*. 1998;19, 2:183-188

Hanada A, Chang MC. Penetration of zona-free eggs by spermatozoa of different species. *Biol Reprod* 1972;6:300–9.

Hanada A, Chang MC. Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to sperm capacitation. *J Reprod Fertil*. 1976; 46: 239-241.

Hermansson U., Axnér E., Holst B.S. Application of a zona pellucida binding assay (ZBA) in the domestic cat benefits from the use of in vitro matured oocytes. *Acta Veterinaria Scandinavica* 2007, 49:28

Liu DY, Baker HWG. Defective sperm–zona pellucida interaction: a major cause of failure of fertilisation in clinical in vitro fertilization. *Hum Reprod* 2000;15:702–8.

Liu DY, Lopata A, Johnston WIH, Baker HWG. Frequency of disordered zona pellucida (zp)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa-binding. *Hum Reprod* 2001;16:1185–90.

McGovern, P.T. The barriers to interspecific hybridization in domestic and laboratory mammals. II. *Hybrid sterility. Br. Vet. J*. 1976;132, 68–75.

McHugh JA, Rutledge JJ, Heterologous fertilization to characterize spermatozoa of the genus Bos. *Theriogenology* 1998;50, 185–193.

Merlo CA, Muñoz E, Dominguez S, Fóscolo M, Scardapane L, de Rosas JC. Seasonal variations in the heterologous binding of viscacha spermatozoa. A scanning electron microscope study. *Biocell* 2005 Dec;29(3):243-51.

Merlo CA, de Rosas JC, Muñoz E, Perez Romera E, Fóscolo M, Domínguez S, Scardapane L, Piezzi RS. Viscacha epididymis and spermatozoa in the reproductive period. *Biocell* 2000;24(3), (Abstract 72/294)

Oehninger S, Franken DR, Sayed E, Barroso G, Kolm P. Sperm function assays and their predictive value for fertilisation outcome in IVF therapy: a meta-analysis. *Hum Reprod Update* 2000;6:160–8

Overstreet JW, Hembree WC. Penetration of zona pellucida of non-living human oocytes by human spermatozoa in vitro. *Fertil Steril* 1976;27:815–31.

Roth TL, Weiss RB, Buff JL, Bush LM, Wildt DE, Bush M.Heterologous in vitro fertilization and sperm capacitation in an endangered African antelope, the Scimitar-Horned Oryx (Oryx dammah). *Biol Reprod* 1998;58:475–82.

Roth TL, Bush LM, Wildt DE, Weiss RB,: Scimitar-Hornerd Oryx (Oryx dammah) spermatozoa are functionally competent in a heterologous bovine in vitro fertilization system after cryopreservation on dry ice, in a dry shipper, or over liquid nitrogen vapor. *Biol Reprod* 1999;60, 493–498.

Soler AJ, Poulin N, Fernández-Santos MR, Cognie Y, Esteso MC, Garde JJ, Mermillod P. Heterologous in vitro fertility evaluation of cryopreserved Iberian red deer epididymal spermatozoa with zona-intact sheep oocytes and its relationship with the characteristics of thawed spermatozoa. *Reprod Domest Anim*. 2008 Jun;43(3):293-8.

M.A. Stoops, J.K. O'Brien, T.L. Roth. Gamete rescue in the African black rhinoceros (Diceros bicornis). *Theriogenology* 2011 Oct 15;76(7):1258-65. Epub 2011 Jul 12.

Taberner E, Morató R, Mogas T, Miró J Ability of Catalonian donkey sperm to penetrate zona pellucida-free bovine oocytes matured in vitro. *Anim Reprod Sci.* 2010;118(2-4):354-61.

Yanagimachi R, Yanagimachi H, Rogers BJ. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of, human spermatozoa. *Biol Reprod* 1976;15:471–6.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. The physiology of reproduction,vol. 1. 2nd *ed. New York: Raven Press*; 1994. p. 189–317.